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HANNA PUHAKKA

Gene Therapy for Vascular Thickening

Doctoral dissertation

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ABSTRACT

Atherosclerosis is the main cause of morbidity and mortality in developed countries. Medication, angioplasty, stenting, and bypass operations are currently the means to relieve the symptoms. However, post-angioplasty restenosis and vein graft stenosis limit the efficacy of these operations. Moreover, the post-operational medication of these patients is still far from optimal.

The aim of this study was to evaluate different gene therapy approaches to treat restenosis and vein graft stenosis. The exact mechanism of pathogenesis of these diseases remains unclear. Tissue inhibitors of metalloproteinase's (TIMPs) are a family of enzymes, which inhibit matrix metalloproteinase (MMP) production. MMPs facilitate cell proliferation and migration and they are found to exist in atherosclerotic lesions and in the neointima. A selective cyclic gelatinase (MMP) inhibitor has been developed using peptide libraries. This inhibitor was synthesized and linked via a poly-lysine spacer to the surface of the adenovirus. *In vitro* and *in vivo* studies were made to test the efficacy of this targeting vector. As a treatment gene we used TIMP-1. It was found that the targeted adenovirus vector had altered tropism. Moreover, it inhibited the neointima formation in a rabbit restenosis model.

Two different pathological steps of neointima formation were affected using two different treatment gene combinations in a rabbit restenosis model. As treatment genes, vascular endothelial growth factor (VEGF) –A, VEGF-C and TIMP-1 were used. VEGFs stimulate the endothelial regrowth. It was found that TIMP-1 gene transfer alone is sufficient for the treatment of restenosis, and gene combinations, used in this study, are not needed.

Also, platelet activating factor acetylhydrolases (PAF-AH) gene therapy was tested in a restenosis rabbit model. PAF-AH inactivates PAF. PAF stimulates secretion of cytokines and smooth muscle cell (SMC) growth. It was discovered that neointima formation was reduced with the help of PAF-AH gene transfer.

A rabbit vein graft model was developed and used in the last study. Vaccinia-virus anti-inflammatory protein 35K is protein which binds all the CC-CK class cytokines. Cytokines are inflammatory mediators which emerge in the vein graft stenosis. Cytokines induce SMC proliferation and migration. TIMP-1 gene transfer was used as a positive control. It was found that 35K gene transfer decreased neointima formation. Macrophage accumulation and the cell proliferation index also decreased with 35K. In other words, inhibiting inflammation processes is important for decreasing vein graft stenosis.

In these studies, several treatment genes and a modified virus vector were used. My results indicate that adenoviral gene transfer is a promising treatment method for the treatment of restenosis and vein graft stenosis. It is concluded that inhibition of restenosis and vein graft stenosis is a multifactorial process and gene therapy is a one way to prevent or delay the process.

National Library of Medicine Classification: QU 107, QW 165.5.A3, QY 60.L3, WG 300

Medical Subject Headings: adenoviridae; arteriosclerosis; coronary restenosis / therapy; cytokines; gene therapy; gene transfer techniques; models, animal; rabbits; tissue inhibitor of metalloproteinase-1; vascular endothelial growth factors

“Of all the things I’ve lost
I miss my mind the most.”

Mark Twain

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I am grateful to Professor Seppo Ylä-Herttuala for giving me the opportunity to work in his group. His enthusiasm and knowledge in this field made these studies possible. I thank also my second supervisor Mikko Turunen for giving me a kick start for my studies. You taught me all the lab and operation techniques I needed. I also prefer Pepsi Max.

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Kuopio, May 2005

Hanna Puhakka

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ABBREVIATIONS

35K	vaccinia virus anti-inflammatory protein
AAV	adeno-associated virus
ACE	angiotensin converting enzyme
Ad	adenovirus
AIDS	acquired immune deficiency syndrome
ANOVA	analysis of variance
ARB	angiotensin receptor blockers
ARDS	acute respiratory distress syndrome
ASAT	aspartyl aminotransferase
AT-1	angiotensin-1
β-gal	betagalactosidase
CABG	coronary artery bypass graft
CRP	c-reactive protein
cDNA	complementary DNA
CAR	coxsacie/adenovirus receptor
CHD	coronary heart disease
CMV	cytomegalovirus
DIC	disseminating intravascular coagulopathy
DNA	deoxyribonucleic acid
EaHy	endothelial tumour cell hybrid
EC	endothelial cells
ECM	extra cellular matrix
eNOS	endothelial nitric oxide synthase
HDL	high density lipoprotein
HIV	human immunodeficiency virus
HSV-tk	herpes simplex virus thymidine kinase
i.a.	intra arterial
I/M	intima/media ratio
i.m.	intra muscular
IEL	internal elastic lamina
i.v.	intra venous
kDa	kilo Dalton
lacZ	β-galactosidase
LAD	left artery descending
LIMA	left internal mammary artery
MCP-1	monocyte chemo attractant protein-1
MMLV	murine leukemia virus
MMP	matrix metalloproteinase
MOI	multiplicity of infection
mRNA	messenger RNA
NZW	New Zealand white
ONPG	o-nitro phenyl β-D-galactopyranoside
OTC	ornitine transcarbamylase
oxLDL	oxidized low density lipoprotein
PAD	peripheral artery disease
PAF-AH	platelet activating factor acetylhydrolase
PBS	phosphate buffered saline
PCI	percutaneus coronary intervention

PEG	polyethylene glycol
PEI	polyethylenimine
PFA	paraformaldehyde
Pfu	plaque forming unit
PIGF	placental growth factor
PTCA	percutaneous transluminal coronary angioplasty
RAASMC	rabbit aortic smooth muscle cells
RITA	right internal thoragic artery
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SDS	sodium dodecyl sulphate
SMC	smooth muscle cell
siRNA	short interfering ribonucleic acid
ss RNA	single stranded ribonucleic acid
Tc	tetracycline
tetO	Tc resestance operator
tetR	Tc resistance repressor protein
TIMP	tissue inhibitor of matrix metalloproteinase
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor

LIST OF ORIGINAL PUBLICATIONS

Thesis is based on the following original publications which are referred to by their Roman numerals (I-IV)

I. Mikko Turunen*, Hanna Puhakka*, Mikko Hiltunen, Juha Rutanen, Olli Leppänen, Anna-Mari Turunen, Ale Närvänen, Andrew Newby, Andrew Baker, Seppo Ylä-Herttuala, *Peptide Re-targeted Adenovirus Encoding Tissue Inhibitor of Metalloproteinase-1 Decreases Restenosis after Intravascular Gene Transfer*, Molecular Therapy vol. 6, no. 3 September 2002 ss 306-312
*equal contribution

II. Hanna L. Puhakka, Päivi Turunen, Juha E. Rutanen, Mikko O. Hiltunen, Mikko P. Turunen, Seppo Ylä-Herttuala *TIMP-1 Adenoviral Gene Therapy Alone is Equally Effective in Reducing Restenosis as Combination Gene Therapy in Rabbit Restenosis Model,* (submitted)

III. Päivi Turunen, Hanna Puhakka, Juha Rutanen, Mikko O Hiltunen, Tommi Heikura, Janne Kokkonen, Marcin Gruchala, Seppo Ylä-Herttuala *Intravascular Adenovirus-Mediated Lipoprotein-associated Phospholipase A_2 Gene Transfer Reduces Neointima Formation in Balloon-Denudated Rabbit Aorta*, Atherosclerosis 179, 2005 ss 27-33

IV. Hanna L. Puhakka, Päivi Turunen, Marcin Gruchala, Christine Bursill, Tommi Heikura, Ismo Vajanto, David R. Greaves, Keith Channon, Seppo Ylä-Herttuala-*Effects of Vaccinia Virus Anti-Inflammatory Protein 35K and TIMP-1 Gene Transfers on Vein Graft Stenosis in Rabbits*, In vivo (in press)

In addition, some unpublished data presented.

TABLE OF CONTENTS

1. INTRODUCTION

Atherosclerosis is a common disease in developed countries causing mortality and morbidity. There are many risk factors associated with it, several of which involve various lifestyle choices, and gender, family history and infections cause atherosclerosis. Medication is the first treatment of choice for atherosclerosis. When medication alone is not sufficient, angioplasty and bypass surgery are reasonable treatment options. The success of these operations is limited by graft stenosis and post-angioplasty restenosis. In addition, the post-operational medical therapy is far from optimal and needs to be efficiently reviewed.

Restenosis and vein graft stenosis are common problems after angioplasty and bypass operations. Vein graft stenosis is a slow process in which the grafts are gradually totally or partly occluded whereas in restenosis the artery occlusion is more rapid. Many gene therapy studies have been carried out with the aim of obtaining a better understanding of the pathogenesis of restenosis and inhibiting the formation of neointima. Drug-eluting stents have become important factors in the clinical treatment of coronary artery patients. In addition, they have shown good treatment results in inhibiting restenosis. However, restenosis remains an important clinical problem. The pathogenesis of vein graft stenosis is somewhat similar to that of restenosis so the same kind of gene therapy methods have been used to decrease the vein graft stenosis.

Gene therapy offers a novel treatment method for inhibiting or delaying the progression of these diseases. In gene therapy, therapeutic genes or a specific gene are introduced into the target tissue. For example, malignant glioma is a devastating brain tumour with no effective treatment. In a randomized controlled study AdvHSV-tk gene therapy with intravenous ganciclovir, improved median survival time from 37.7 to 62.4 weeks (Immonen et al., 2004). The development of new viral vectors and delivery methods has given us the means to achieve longer gene expression times in the target tissue and improve results.

With a seemingly great future ahead, gene therapy has also experienced setbacks. In 1999 the first death during a gene therapy experiment, resulting clearly form gene therapy itself, was reported. An 18-year old boy developed disseminating intravascular coagulupathy (DIC) and acute respiratory distress syndrome (ARDS) after adenoviral gene transfer (Lehrman, 1999). Thus, cytotoxity and the systemic spread of the vector are problems which need to be overcome. Targeting viral vectors to specific cell types is one way to inhibit this problem. In addition, combination studies with gene cocktails could lead to better results. Affecting two or more pathological steps of pathogenesis of these disease long-term results could be achieved.

2. REVIEW OF THE LITERATURE

2.1. ATHEROSCLEROSIS-RELATED DISEASES

Atherosclerosis is the most common disease in developed countries. In Finland, it is the biggest cause of mortality and morbidity. Development of the atherosclerosis begins in childhood but the symptoms usually emerge much later (Stary, 2000). Atherosclerosis is a systemic disease and the development of atherosclerosis causes coronary heart disease, peripheral vascular disease, carotid artery disease, and abdominal aorta aneurysm among other problems (Dormandy and Rutherford, 2000; Kannel, 1986). Being a progressive disease it causes growing obstructive lesions to the vessel wall which will later on develop cardiovascular symptoms.

The main risk factors for the development of atherosclerosis are high blood pressure, high levels of cholesterol in the blood, smoking, diabetes, kidney disease, failure of anticoagulant pathways, male gender, low physical activity, family history of atherosclerosis and infections (Ross, 1999). It is thought that the severity of atherosclerosis correlates with the number of risk factors. Therefore, preventing atherosclerosis should involve dietary restrictions with lipid-lowering, high blood pressure-lowering and anti-thrombotic medication. In the case of advanced disease, percutaneus and surgical operations have been combined with medication to relieve the symptoms of atherosclerosis.

2.1.1. Pathogenesis of atherosclerosis

There are three main hypotheses why atherosclerosis develops. One hypothesis is that there is damage/injury (e.g. inflammation, viruses, oxidized low density lipoprotein (oxLDL), stress) in the endothelia (Ross, 1986). The other hypothesis is that the insulation of lipids to the intimal layer of the vessel wall disturbs the normal structure and metabolism of the vessel (Steinberg et al., 1989). Also, vascular infections have been one suggested cause of atherosclerosis (Murdoch and Finn, 2000).

As mentioned earlier, atherosclerosis is initiated in response to the factors which cause injury or dysfunction to the endothelia (table 1 presents the pathological steps of atherosclerosis, the normal structure of the vessel is presented in figure 1 A). This injury allows lipids to leak into the sub-endothelial space. In the early stages of atherosclerosis LDL binds to the proteoglycans of the vessel wall leading to a locally increased concentration of LDL (Boren et al., 1998). Versican is the principal proteoglycan in the blood vessel and it is found to be

upregulated in the atherosclerotic leasions. Also, versican appears to contribute to intimal expansion and lesion progression (Wight and Merrilees, 2004).

The LDL bound to the vessel wall is furthermore modified by oxidation and glycation. Oxidized LDL damages endothelia and attracts monocytes and T-cells (Steinberg, 1997). Also, the injury in the endothelium attracts the chemochines and permits the monocytes, T-cells and platelets to enter the subendothelial space (figure 1 B) (Reape and Groot, 1999). In the subendothelial space, the monocytes transform into the macrophages which accumulate modified lipids which in turn transform into foam cells. Together with the T-cells the foam cells form the fatty streak in the vessel wall (Stary et al., 1995).

	What happens first?	**This step is called**	**What next?**
1st phase	*Injury* to endothelia allows lipids to flow to subendothelia	***OxLDL bindig*** to proteoglycans	oxLDL cause further damage to endothelia and attract monocytes and T-cells to subendothelial space
2nd phase	macrophages accumulate lipids becoming *foam cells*	***fatty streaks***	lesion progresses
3rd phase	*a lipid core* containing calcium chosterol and lipids is formed	***atheroma***	fibroatheroma is formed when lesion develops further
4th phase	SMCs migrate and proliferate to intima, the leasion develops	***plaque***	symptoms of *angina pectoris* and plaque rupture if vulnerable plaque

Table 1. The different steps of atherosclerosis. The progression of atherosclerosis is described in different rows.

Fibroatheroma is the intermediate lesion (table 1). Upon disease progression, the normal structure of the intima is replaced by the necrotic core. The necrotic core contains cholesterol crystals, calcium and extracellular lipids. As a further development, the lipid rich core becomes surrounded by connective tissue and forms fibrous plaques. Macrophages, smooth muscle cells and endothelial cells secrete chemokines and growth factors which attract more monocytes and growth factors from the media to the intima (Tsukada et al., 1986). During this phase the lesions start to protrude into the arterial lumen (figure 1 C). This also damages the endothelia and exposes the vessel to haemorrhage or thrombus formation (Stary et al., 1995). It has been observed that SMCs cultured from atherosclerotic coronary atherectomy specimens proliferate more slowly and demonstrate increased apoptosis as compared to SMCs from normal vessels (Bennett et al., 1998). This

process may lead to plaque destabilization since apoptotic and necrotic cells have been detected in atherosclerotic plaques with a recent history of rupture. Also, SMC apoptosis can be observed in the fibrous cap (Bjorkerud and Bjorkerud, 1996).

Symptoms of stable ischemia emerge as the result of gradual occlusion of the vessel, whereas the symptoms of unstable ischemia are caused by rupture of the plaque. Lesions, which contain a large lipid core and a thin lipid cap, are called vulnerable plaque whereas the stable plaque is the opposite, containing a small lipid core and a thick fibrous cap. Usually, vulnerable plaque is more prone to rupture causing symptoms of angina pectoris. Autopsies have revealed that ruptured plaques typically contain a large necrotic core with a fibrous cap infiltrated by macrophages and T-cells and the SMC levels also tend to be low in the ruptured plaques. Also, it has been found that matrix metalloproteinase (MMP) levels, especially MMP-8 and MMP-9, are increased in the shoulder regions of vulnerable plaques (Molloy et al., 2004; Brown et al., 1995). Moreover, it has been found that vulnerable plaques have a higher temperature than stable plaques and they also express lower pH levels (Madjid et al., 2002; Naghavi et al., 2002). Indeed, it is suggested that patients with cholesterol levels >5, high levels of C-reactive protein (CRP) or elevated levels of other biomarkers MMP-9 or monocyte chemoattractant protein-1 (MCP-1), should be considered at high risk of vulnerable plaques. Also, stenosis > 50% with necrotic cores > 120° would strongly suggest unstable plaque morphology (Kolodgie et al., 2004; Galis, 2004).

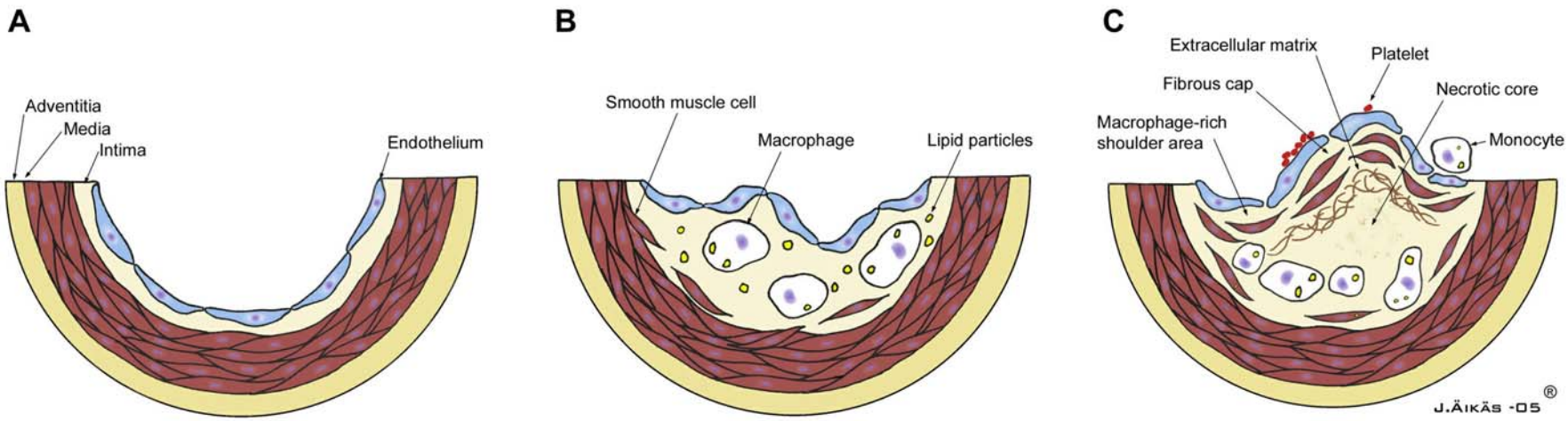

Figure 1. A) The structure of the normal vessel. B) The endothelial damage allows the lipids to leak into the subendothelial space attracting LDLs. Medial SMCs proliferate and migrate towards the intima. C) Fibroatheroma, the lesion protrudes into the lumen and the fibrous plaque develops. Shoulder regions containing a lot of macrophages and T-cells are prone to rupture.

2.1.2. Pathogenesis of restenosis

Angioplasty is the most common invasive treatment method of atherosclerosis. At present, angioplasty is widely used for the treatment of acute infarctation. Angioplasty presents two main temporarily related concerns: abrupt closure and delayed restenosis. The reduction of the luminal size after an intravascular procedure is called restenosis. Studies show that intravenous operations cause injuries to the endothelium, stretch the artery, and locally tear the neointimal plaque and media (Serruys et al., 1988). As a result, in studied animal models, restenosis has been seen to resemble wound healing (figure 2). Interestingly enough, the results from the animal models appear to mimic human histopathological findings.

The rate of restenosis correlates with factors following treatments including residual stenosis, irregularity of the lumen surface and relative rate of blood flow. Despite technical improvements, restenosis after conventional balloon angioplasty occurs in 30 - 60% of cases within six months of the procedure (Serruys et al., 2002). Risk factors of restenosis include diabetes, lesion location (left artery descending e.g. LAD is more susceptible to restenosis), residual stenosis, increased number of stents, post-prandial increase in remnant like particles, cholesterol (RLP-C) concentrations and increased leptin levels (Schwartz and Henry, 2002; Oi et al., 2004; Piatti et al., 2003).

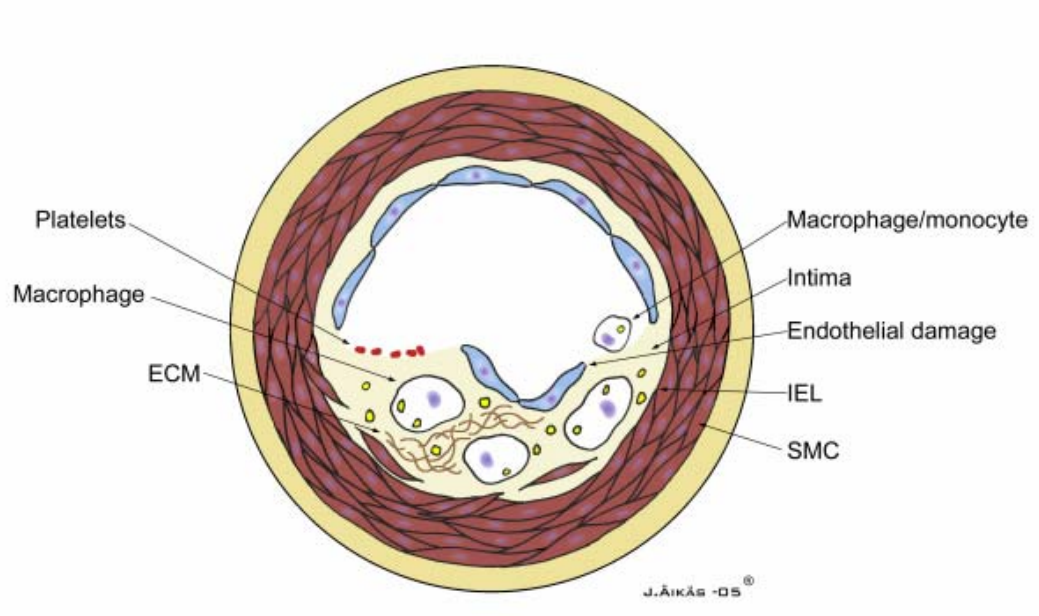

Figure 2. Pathogenesis of restenosis. The balloon denudation damages the endothelia. Neointima proliferation can be seen even before the thrombus is absorbed. SMCs start to migrate towards the intima-layer from the media-layer. Also, the cell infiltration from the damaged endothelia initiates the inflammation process. Internal elastic lamina (IEL), smooth muscle cell (SMC), extracellular matrix (ECM).

In animal models, deendothelialization after angioplasty exposes the subendothelial structures to circulating blood elements causing thrombotic and inflammatory phases to emerge. At first, a rapid formation of thrombus is detected. The platelet thrombus can grow large enough to occlude the vessel and this process is called abrupt closure. The first wave of SMC apoptosis is initiated hours after angioplasty, resulting in a decrease of cellularity of the vessel wall. Walsh et al suggested that greater wound healing might be provoked by releasing cytokines in the response to overcome the cellular deficit (Walsh et al., 2000).

The final phase of wound healing is regrowth of the endothelial layer on the surface of the thrombus. The regrowth can also be incomplete (Serruys et al., 1988; Nikol et al., 1996). At the same time an intense cellular infiltration occurs. Infiltrating monocytes and lymphocytes release growth factors causing SMCs to migrate to the intima (Schwartz, 1998). The cell proliferation process is initiated by the infiltrating cells. Actin-positive cells colonize the residual thrombus and progressively proliferate towards the media, at the same time reabsorbing the thrombus. The thrombus is replaced by neointimal cells. This completes the healing process (Schwartz and Henry, 2002). Also, the rat models have shown that the second wave of apoptosis occurs at this stage and it is mainly confined to the SMCs of the developing neointima. It has been thought that the second wave of apoptosis limits the leasion size. Rates of neointimal SMC death and proliferation are in equilibrium from two weeks onwards (Walsh et al., 2000). Arterial healing in animals has been found to be similar to that in humans.

In animal models SMCs are mostly derived from pre-existing media and they likely contribute to the intimal hyperplasia. The highest proliferation activity of SMCs occurs a couple of days after the injury. SMCs are surrounded by and embedded in ECM proteins including elastin, collagens and proteoglycans. Actually, intimal thickening requires the controlled degradation of the ECM and the activation or the release of growth factors. Therefore, ECM remodelling is an ongoing process, lasting up to several weeks and months. Constructive arterial wall remodelling has emerged as the dominant determinant of lumen narrowing after angioplasty. The molecular basis of the arterial wall shrinkage has not been defined, but studies have suggested that the changes in the arterial wall geometry must involve reorganization of the ECM (Jarvelainen et al., 2004; Wight and Merrilees, 2004). It has been shown that decorin, a small proteoglycan, inhibits the accumulation of ECM (Giri et al., 1997). Recently, Järveläinen and colleagues showed that over-expression of decorin enhanced contraction *in vitro* suggesting that decorin stabilizes the ECM (Jarvelainen et al., 2004).

There is strong evidence that inflammatory response correlates significantly to the degree of arterial injury, as the inflammatory reaction triggers a cascade of thrombotic and hyperplasic sequel (Kornowski et al., 1998; Schwartz and Henry, 2002). A recent study suggests that the ubiquitin–proteasome system of intracellular protein degradation is implicated as a key player in restenosis (Meiners et al., 2002). This system regulates

mediators of proliferation, inflammation, and apoptosis that are fundamental mechanisms for the development of restenosis.

2.1.3. Pathogenesis of vein graft stenosis

Vein grafts are the most commonly used conduits for aortocoronary (CABG) and peripheral bypass grafting. However, arteria grafts (left internal mammary artery (LIMA), right internal thoragic artery (RITA), radial and gastroepiploica) are also being used in CABG. Bypass grafts improve anginal symptoms in patients with coronary artery disease, but the main problem still lies with long term results. Despite the success of the CABG during the first year after bypass surgery up to 15% of venous grafts occlude. Between 1 and 6 years the graft attrition rate is 1% to 2% per year, and between 6 and 10 years it is 4% per year. By 10 years after surgery only 60% of vein grafts are patent and only 50% of patent vein grafts are free of significant stenosis (Motwani and Topol, 1998; Fitzgibbon et al., 1996).

Studies show that central to vein graft failure is the formation of the neointima with subsequent atherosclerosis. Actually, there are three main factors which contribute to the pathogenesis of vein graft stenosis: thrombosis, neointima formation and atherosclerosis. Thrombotic occlusion is the major problem during the first weeks after a successful operation (Motwani and Topol, 1998). Between 3% and 12% of saphenous vein grafts occlude, with or without symptoms, within the first month after bypass surgery (Fitzgibbon et al., 1996).

The graft thrombosis is caused by a combination of alterations in the vessel wall, changes in blood rheology, and altered flow dynamics (Cox et al., 1991; Motwani and Topol, 1998). The vein is always subjected to focal endothelial damages, no matter how subtle the surgical operation is. Also, endothelial damage activates the coagulation cascade. The Blood flow rate itself, as mediated by surface shearing forces across the endothelium, has been identified as an important regulator of both the biochemical and the morphological changes that occur during early graft remodelling (Allaire and Clowes, 1997).

Intimal hyperplasia in vein grafts has been shown to develop due to early vein graft injury mainly near to the anastomosis site (Davies and Hagen, 1995). Autopsies show that the process of neointima formation in vein graft stenosis is quite similar to that in restenosis. In addition, in vein graft stenosis intimal hyperplasia occurs after endothelial regeneration. It involves the replication of SMCs which migrate to the intima where they continue to proliferate and secrete ECM proteins (Davies and Hagen, 1995). SMC proliferation is induced by

cytokines and growth factors released from endothelial cells, platelets and macrophages. Later, synthesis and deposition of the extracellular matrix by activated SMCs leads to a progressive increase in intimal fibrosis and a reduction in cellularity (Allaire and Clowes, 1997). Also, the loss of the vasa vasorum blood supply may promote a continuing cycle of ischemia and fibrosis. SMCs in the media of normal adult arteries proliferate at a very low rate. However, they can turn on rapidly to a proliferative state in response to appropriate stimuli (Majesky et al., 1987). A transition of arterial SMCs to an active state has been implicated in the failure of human vascular reconstructions after arterial bypass grafting as well as following angioplasty.

Intimal hyperplasia generates the foundation for the development of graft atheroma. However, atherosclerosis is the underlying process due to which the graft stenosis eventually occurs. Histological studies have shown that vein graft atheroma has more foam cells and inflammatory cells than native coronary atheroma. This appears to be similar to experimental models of immune-mediated atherosclerosis (Ratliff and Myles, 1989). The data from autopsies suggests that vein graft atherosclerosis in vein grafts tends to be diffuse with a poorly developed or absent fibrous cap. The atheromas present only a very little calsification (Motwani and Topol, 1998).

2.2. CURRENT TREATMENT METHODS FOR RESTENOSIS AND VEIN GRAFT STENOSIS

2.2.1. Medication

Medication is the first-line treatment choice for atherosclerosis, and for the prevention of restenosis and vein graft stenosis. Antithrombotic agents are the primary treatment method for the prevention of thrombosis after angioplasty or vein graft operations. Aspirin is shown to have an anti-thrombotic effect and studies have indicated that the use of aspirin reduces the risk of myocardial infarctation (Manson et al., 1992). Current evidence from clinical trials favours the use of aspirin or clopidogrel as first-line agents for the majority of patients with vascular disease. The CAPRIE study suggested that long-term administration of clopidogrel to patients with atherosclerotic vascular disease is more effective than aspirin in reducing the combined risk of ischemic stroke, myocardial infarction, or vascular death (1996b). Further, Akbulut et al showed that long-term administration of clopidogrel after PCI reduces neointimal formation and major clinical events in patients with no systemic inflammatory response (Akbulut et al., 2004). Also, Glycoprotein (GP) IIb/IIIa inhibitors seem to have a potential plaque stabilizing effect. Since patients with diabetes have increased populations of platelets, GP IIb/IIIa inhibitors are recommended for diabetic patients with acute coronary syndrome (Roffi et al., 2001). Combination antiplatelet therapy is being evaluated as an effective option for those patients who experience

recurrent events on a single antiplatelet agent (Zusman et al., 1999; Mehta and Yusuf, 2000; Saw et al., 2004). In addition, the use of aspirin, ticlopidine, clopidogrel, aspirin combined with clopidogrel, and aspirin combined with dipyridamole are effective in preventing recurrent vascular events among various subgroups of patients with vascular disease (1994; Tran and Anand, 2004).

A reduction of LDL levels using lipid lowering drugs has resulted in improved endothelial functioning (Anderson et al., 1995). Lipid-lowering agents also alter intimal plaque stability in a manner which is endothelium-independent. It is believed that lipid-lowering may therefore influence the matrix degradation cascade which appears active in macrophage-rich areas of the atheroma, as well as promote mechanical stability within the plaque (Treasure et al., 1995). After aggressive adjustment of lipid levels, the decrease in CRP levels was independently and significantly correlated with the rate of progression of atherosclerosis (Ridker et al., 2005; Nissen et al., 2005). In the two year follow up, the REGRESS study showed significantly lower restenosis rates with patients treated with statins (Mulder et al., 2000). Also, atorvastatin has showed decreased inflammatory response after PCI (Gaspardone et al., 2002). Other cholesterol-lowering drugs (bile acid sequestrants, niacin, plant stanols, and fibrates) are much less effective in lowering LDL and are much less well tolerated but may be useful when combined with statins. A novel class of agents, cholesterol transport inhibitors such as ezetimibe, have recently become available. Also, plant stanol is an effective strategy in the management of hypercholesterolemic patients. These and other new agents hold promise for helping to achieve LDL goals when used in combination regimens with statins (Stein, 2003).

Oxidation of LDL increases its atherogenic potential. Antioxidants may promote plaque stabilization through their inhibition of LDL oxidation and reduction of matrix degeneration within the plaque. Treatment with antioxidants has been shown to reduce intimal lesions after balloon injuries in animals (Nunes et al., 1995). In the CLAS study less coronary artery lesion progression was found with a daily intake of vitamin E (Hodis et al., 1995). However, a recent study shows that long-term oral vitamins C and E do not reduce atherosclerosis, improve endothelial dysfunction, or reduce LDL oxidation (Kinlay et al., 2004). Probucol has been shown to reduce restenosis in several clinical trials, but its effect on HDL cholesterol and QT intervals have limited its use (Tardif et al., 1997; Rodes et al., 1998; Yokoi et al., 1997). On the other hand, Probucol derivate, with similar antioxidant properties, has shown promising results in clinical studies reducing restenosis and also with decreased risk in arrhythmias (Tardif et al., 2003). Also, there has been continuous discussion about whether diets with a daily small intake supplemented with antioxidants (e.g. wine, fruits, chocolate) could influence plasma lipid metabolism and plasma antioxidant capacity and that therefore, daily use may be beneficial in the prevention of atherosclerosis and coronary heart disease (Puddey and Croft, 1999; Mursu et al., 2004).

However, more research is needed before these compounds can be conclusively considered dietary anti-oxidants with nutritional benefit.

Nitrates are the most used class of coronary medication. Nitro-glycerine and the long-acting nitrates are beneficial in stable and unstable angina pectoris and acute myocardial infarction and as adjunctive therapy in congestive heart failure. Nitro-glycerine compounds relax vascular smooth muscle, decreasing preload and afterload, and producing venous, arterial, and arteriolar dilatation (Rapaport, 1985). Also, nitrates improve exercise performance in stable angina pectoris.

Hemodynamic forces may cause disruption of a vulnerable plaque. β-adrenergic blockers and angiotensin converting enzyme inhibitors may reduce the incidence of acute coronary symptoms by reducing the hemodynamic forces that promote the plaque rupture. Also β-adrenergic blockers have been shown to have a secondary preventive function in reducing incidences of reinfarctation (Yusuf et al., 1985). Beta blockade has resulted in a 40% improvement in survival rate when taken overall. Although the use of beta blockade after acute myocardial infarctation has a major prognostic importance, Gottlieb et al have suggested that the specific beta blocker chosen will have a minor effect on mortality (Gottlieb and McCarter, 2001).

Angiotensin converting enzyme (ACE) inhibitors have been shown to reduce cardiac events in people with known coronary artery disease (Konstam et al., 1992; Collins et al., 1990). Both the HOPE and the EUROPA studies provided evidence suggesting that patients with stable cardiovascular disease or diabetes (plus one additional risk factor) should be treated with an ACE inhibitor (Fox, 2003; Dagenais et al., 2001). The CHARM study suggested that angiotensin II receptor blockers (ABR)s have clinically important effects on haemodynamics and left-ventricular remodelling when added with ACE inhibitors in patients with chronic heart failure (McMurray et al., 2003). When considering the difference in treatment of hypertonia ACE inhibitors, calcium channel blockers (CC blockers) and diuretics, the ALLHAT study recommended diuretics to be the first-step choice for antihypertensive therapy (2002). Whereas when calcium channel blockers and ACE inhibitors are compared, CC blockers are recommended to patients with normal blood pressure and coronary artery disease resulting in reduced numbers of cardiac events (Nissen et al., 2004).

Levels of plasma C-reactive protein (CRP) have been found to be significantly higher and more prolonged in patients with in-stent restenosis compared to patients without restenosis (Gottsauner-Wolf et al., 2000). Similar findings were reported lately in a series of patients with stable angina that underwent PCI (Almagor et al., 2003). Therefore, treatment of inflammation after percuntaneus interventions might be beneficial (Toutouzas et al., 2004). Moreover, the IMPRESS study showed that prednisolon treatment in patients with high CRP levels

reduced restenosis (Versaci et al., 2002). Also, the OSIRIS trial showed that 10 day oral treatment with Sirolimus (rapamycin) and loading before PCI resulted in an improvement in the restenosis rate (Hausleiter et al., 2004).

2.2.2. Angioplasty and stenting

Angioplasty is routinely used as a percutaneus treatment for atherosclerosis and restenosis. It has also been a routine treatment for acute infarctation mainly when antiplatelet treatment is not effective for the symptoms. When compared to medical therapy angioplasty reduces the frequency of anginal episodes (1990). Angioplasty compresses and breaks the plaque and increases the size of the lumen for the blood flow. Also, it makes medial dissections which make luminal expansion more persistent (Bittl, 1996). The drawback, however, is the restenosis which affects 30% to 60% the patients within six months of the procedure (Serruys et al., 2002).

Studies suggest that PTCA is indicated if the desired level of anginal relief and physical activity cannot be achieved with medical therapy alone, but that prophylactic PTCA cannot be recommended for the treatment of coronary artery stenosis in the absence of angina or ischemia (Rihal et al., 2003). The same study indicated that in the presence of an anatomic stenosis, when comparing PTCA to medication, balloon angioplasty is indicated for symptom improvement. They also found that balloon angioplasty does not prevent death or myocardial infarction. Additionally, Rihal et al suggested that PTCA is associated with a greater need for subsequent CABG surgery.

Stents have primarily been used to prevent post-angioplasty restenosis. The intracoronary stent improves the long-term minimum luminal diameter and decreases the rate of restenosis by eliminating the arterial remodelling. However, the in-stent restenosis remained a significant clinical problem featuring the occlusion of the stented vessel area (Kornowski et al., 1998). A bare metal stent possesses little biologic activity against neointima formation and stimulates neointimal thickening when compared to angioplasty. Morphology after stenting demonstrates thrombus formation and acute inflammation with subsequent neointimal growth. Inflammation is associated with medial injury and lipid core penetration by stent struts. Medial damage and stent over-sizing are associated with neointimal growth (Schwartz and Henry, 2002).

Earlier it was found very difficult to treat in-stent restenosis, because the stent doesn't allow dilatation. The risk of restenosis is nearly 80% in dilated in-stent restenosis (Bittl, 1996). The coated stents have recently been a

successful treatment method for in-stent restenosis. Today, the number of agents under preclinical and clinical investigation has increased considerably, including drugs such as Paclitaxel, Sirolimus, Tacrolimus, Everolimus and Dexamethasone. Sirolimus (RAVEL and SIRIUS trials) or Paclitaxel (TAXUS trials) coated stents have shown persistent reduced restenosis levels in clinical studies (Regar et al., 2002; Fattori and Piva, 2003; Lansky et al., 2004; Morice et al., 2002; Fajadet et al., 2005). Kastrati et al suggested that when sirolimus- and paclitaxel-eluting stents are compared in high-risk patients, sirolimus-eluting stents may be more effective in reducing in-stent restenosis (Kastrati et al., 2005).

However, there remain concerns with drug-eluting stents, e.g. late thrombosis likely due to inhibition of reendothelialization, localized hypersensitivity reactions, decreased efficacy in diabetic patients and that the underlying atherosclerotic process is not targeted (Deng et al., 2004; Moses et al., 2003). In a recent multicenter study, increased rates of stent thrombosis, myocardial infarctation, and cardiac death was associated with the QuaDDS (SCORE trial using paclitaxel derivate) stent showing an unacceptable safety profile (Grube et al., 2004). The angiographic indications of potential anti-restenotic effects were still remarkable and similar results with no adverse effects have also been shown in the RAVEL, SIRIUS and TAXUS trials (Fajadet et al., 2005; Grube et al., 2002) suggesting that the local application of anti-proliferative agents delivered by coronary stents is one of the most promising techniques for the treatment of coronary lesions.

There have also been some studies concerning intra-coronary radiation therapy and its efficacy in reducing in-stent restenosis (Janicki, 2003; Leon et al., 2001). The drawback of these studies has usually been the incidence of stent thrombosis despite the optimization of pre- and post procedural factors (Serruys et al., 2004; Waksman et al., 2000a). On the other hand, several studies have also shown quite the opposite, being effective in reducing in-stent restenosis (Teirstein et al., 2000; Waksman et al., 2000b; Verin et al., 2001). Moreover, Waksman et al have shown that the incidence of late thrombosis in brachytherapy was reduced by extending the duration of dual antiplatelet therapy by 6 to 12 months (Waksman et al., 2002). Therefore, more studies are needed to value the effectiveness between brachytherapy and drug-eluting stents in reducing restenosis.

2.2.3. Bypass grafting

Coronary artery bypass grafting (CABG) gives a symptom-free period for patients with severe coronary heart disease. The three potential reasons to recommend myocardial revascularization are (1) to alleviate symptoms of myocardial ischemia, (2) to reduce the risks of future mortality, and (3) to treat or prevent morbidities such as myocardial infarction, arrhythmias or heart failure. The results of the operation are as effective as angioplasty or stenting (Taggart, 1993; 1996a; Hoffman et al., 2003). PTCA is preferred as a treatment option, when the patient has a single-vessel disease (Berger et al., 2001). When a meta-analysis was made between CABG and medication, it was found that the mortality benefits of CABG surgery are proportional to the baseline patient risk. Also, it was found that CABG surgery does not reduce the overall incidence of nonfatal myocardial infarction and that CABG is effective for symptom improvement (Rihal et al., 2003).

CABG surgery is likely preferred for high-risk patients such as those with left main, severe three vessel disease, or diffuse disease, severe ventricular dysfunction, or diabetes mellitus. When compared to PTCA both PTCA and CABG provide good symptom relief (Hoffman et al., 2003). However, repeat procedures are required more frequently after PTCA than after CABG. In the seven year follow up of the BARI study, there was a statistically significant survival advantage for patients randomized to CABG when compared with PTCA (2000). Although CABG improves both survival and anginal symptoms in patients with severe coronary artery disease, the problem of vein graft failure continues to limit its long-term success. Arteria grafts have a better outcome in the graft stenosis rate than venous grafts. A recent study shows that radial-artery grafts are associated with a lower rate of graft occlusion at one year than are saphenous-vein grafts (Desai et al., 2004). Also, the use of the LIMA results in increased survival when compared to revascularization with vein grafts. Internal mammary grafts demonstrate increased patency compared to vein grafts; approximately 90% after 5 years and 83% after 10 years (Eagle et al., 1999).

Bearing in mind that the venous restenosis process is slower than the arterial, still more than 50% of coronary venous bypass grafts fail within 10 years of implantation, making vein graft failure the leading indication for repeat CABG (Grondin et al., 1984). On the other hand, numerous studies show that secondary prevention after CABG in terms of quitting smoking, treatment of hyperlipidemia and hypertension is far from optimal and can be improved considerably (Irving et al., 2000; Allen et al., 2001; 2001).

2.3. GENE THERAPY FOR RESTENOSIS AND VEIN GRAFT STENOSIS

Gene therapy is a relatively new treatment method introducing therapeutically important genes in the somatic cells of patients for the treatment of an acquired or inherited disease. Gene therapy can be used in various ways. One method is to over-express proteins which are therapeutically useful. Another method is to correct a genetic defect via gene replacement or gene repair. Additionally, the silencing of genes involved in the pathological processes may be desirable. The acute blockade of gene transcriptions can be achieved by treatment with short single-stranded antisense oligodeoxynucleotides, ribozymes, and more recently, using RNA interference technology. These molecules inhibit the synthesis of proteins by hybridizing in a sequence-specific complementary fashion to target mRNA.

Double-stranded decoy oligonucleotides containing DNA consensus binding sequences have been used to inhibit transcription factor DNA binding. The decoy is usually delivered in excess, sequestering the target transcription factor and rendering it incapable of binding to the promoter region of the target gene. Ribozymes are known to catalytically cleave specific target RNA leading to degradation, whereas antisense decoys inhibit translation by binding to mRNA sequences on a stoicheometric basis. Ribozymes knock down selectively targeted genes in human tumours grown *in vivo* but delivery issues of these therapeutic anti-genes limit clinical utility. Short interfering RNA (siRNA) is at present the fastest growing sector of the anti-gene field for target validation and therapeutic applications. The siRNA field may have an opportunity to impact clinical therapy faster than antisense and ribozymes if the scientists can overcome anti-gene limitations.

A carrier molecule, called a vector, must be used to deliver the therapeutic gene/genes to the patient's target cells which then express the encoded proteins. Many constructs have been developed to achieve the acquired effect (table 2). However, there have been drawbacks with transgene size, production, expression time and toxicity. Non-viral vectors e.g. plasmid DNA, do not have those problems. The main problem with plasmid DNA is low efficacy. Currently, the most commonly used vector is an adenovirus that has been genetically altered to carry a transgene.

Atherosclerosis, restenosis and vein graft stenosis are attractive targets for gene therapy because they affect a large number of people and animal models are relatively easy to establish. Vascular gene therapy is nowadays mainly focused on inhibiting SMC proliferation and migration, increasing re-endothelialization and inhibiting inflammation and oxidization. Gene transfer can be done in different ways depending on the animal

model, treated disease and on the treatment mechanism of the gene. The most common method is intravascular gene transfer which can be done using catheters. Intravascular gene transfer leads to the transduction of SMCs and endothelia and it can be done during angioplasty and stenting (Ylä-Herttuala and Martin, 2000).

Vector	*in vivo* efficacy	transgene size	titers	transduction of non-dividing cells	expression time	drawbacks
Adenovirus	+++	+	+++	yes	transient, 2-4 weeks	very immunogenic, inflammatory reactions
Adeno-associated virus	++	+	+++	yes	stable, several months	very immunogenic, difficult production
MMLV Retrovirus	+	++	+	no	stable, over 6 months	-
Lentivirus	+	++	++	yes	stable, over 6 months	toxic at high titers
Baculovirus	++	+++	+++	yes	transient	immunogenic
Herpes simplex virus	+	+++	+	yes	long-lasting/ latent	very toxic
Non-viral	+	+++	-	yes	transient	+/-

Table 2. Properties of gene transfer vectors. +++ indicates efficient quality, ++ indicates moderate, and + poor, - indicates low titers or no drawbacks and +/- indicates that there are some drawbacks but not severe.

2.3.1. Gene transfer vectors and gene delivery

Adenoviruses are the most commonly used gene transfer vectors. There are various types of adenoviruses but serotype-5 is most commonly used for gene therapy. Adenoviruses are capable for transducing dividing and non-dividing cells. They enter cells via the coxacie-adenovirus receptor (CAR) and integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ which mediate attachment and internalization (Bergelson, 1999). CAR is mainly present in hepatocytes, the myocardium and in epithelial cells, whereas the endothelium and SMCs express it only in low levels (Biermann et al., 2001). Adenoviral vectors can be produced in high titers, and they can be used for an efficient but dose dependent gene transfer in the target tissue. Adenoviruses offer a transient gene expression from two to four weeks which is suitable for the treatment of diseases such as restenosis and vein graft stenosis where the pathological events occur soon after the injury (Hiltunen et al., 2000a; Laukkanen et al., 2002). The side-effects of adenoviruses vary from flu to gastroenteritis.

Adenoviruses are used for oral vaccines and gene therapy. It has been noticed that adenoviruses are safe for humans and infections are not associated with malignancies. It has also been demonstrated that adenoviral-mediated gene transfer is feasible and well-tolerated by human peripheral arteries (Laitinen et al., 1998). The problems with adenoviruses arose with their immunogenity and pro-inflammatory effects. Immunostimularity properties may limit repeated gene transfers or the use of high titers of adenoviruses.

Adeno-associated viruses (AAV) are replication defective parvoviruses. Currently there are eight different human AAV serotypes of which the most commonly used is AAV2. AAVs are capable for transfecting both dividing and non-dividing cells (Summerford et al., 1999). They enter cells via heparin sulphate proteoglycan using co-receptors such as $\alpha_v\beta_5$ integrin and human fibroblast growth factor 1. Transduction can be done to the various kind of cells i.e. SMCs, the skeletal muscle, the retina and the central nervous system (Snyder et al., 1997). The efficiency of AAVs depends on serotypes but gene expression lasts for several months. AAVs are also immunogenic but the wild-type virus is considered non-pathogenic and it has not been associated with any human disease. AAVs are promising vectors for gene therapy due to their long lasting transgene expression. Therefore, they can be used to obtain sustained therapeutic effects in, for example, the myocardium or skeletal muscle. The problems with AAVs include their difficult production and their small transgene capacity (Dong et al., 1996).

Murine Leukaemia Virus (MMLV) retroviruses have the ssRNA genome. MMLV retroviruses do not cause severe immunological reactions. However, some malignancies are caused by retrovirus infections due to the integration of the transgene preferentially to active chromatin (Miller, 1992; He et al., 2002). The infection is done through the target cell surface receptor with the interaction of an envelope protein. MMLV retroviral vectors cannot transfect non-dividing cells because they need replication for the entry into the nucleus (Boulikas, 1998). MMLV transduction leads to stable transgene expression. In cancer gene therapy they have an advantage in transfecting rapidly dividing tumour cells when compared to other viral vectors. The limiting factors with the use of retroviruses are the low titers, low transduction efficacy and inability to infect non-dividing cells. These problems have been overcome by pseudotyping MMLV retroviruses with VSV-6 envelope proteins resulting in higher titers and broader tropism.

Lentiviruses (such as HIV) belong to the family of retroviruses. They are integrating viruses and transduce both dividing and non-dividing cells (Trono, 2000). They infect several kinds of cells in the body i.e. CD4 positive T-cells and monocytes. Parental lentiviruses can effectively disable the immune system and destroy its capability to fight disease, which eventually leads to AIDS. Ex vivo studies have shown that lentiviruses also

efficiently infect pancreatic islets, brain tissue and liver and muscle cells. Transgene expression with lentiviruses is long lasting. Greater than six month expression times have been reported (Debyser, 2003).

Baculoviruses are a group of insect viruses. They enter the cell by absorptive endocytosis. Cellular surface molecules for attachment and entry are not known, but studies with target cells suggest that the attachment molecule is a common cell surface component. Baculoviruses transduce various dividing and non-dividing cells. They produce a transient gene expression that may last weeks. Baculoviruses contain nearly all genes of the native genome of the virus which makes it exceptional compared to other viruses. However, baculoviruses have a safety advantage when compared to other viral vectors, because they do not replicate in mammalian cells (Huser and Hofmann, 2003).

Herpes simplex viruses (HSV) are DNA viruses and they cause infections in humans from venereal diseases to meningitis. They do not integrate into the host cell genome. HSV can infect several cell types including lung, liver and muscle cells and they can also transfect non-dividing neural cells. As a result, HSVs have been used in the treatment of Parkinson's disease, cerebral ischemia and malignant gliomas (Marconi et al., 1996). The drawback however, is toxicity and inflammatory reactions in many cell types. On the other hand, cytotoxicity might be useful for cancer gene therapy.

Besides virus-mediated gene-delivery systems, there are several nonviral options for gene delivery. The simplest method is the direct introduction of therapeutic DNA into target cells. This approach is limited in its application because it can only be used with certain tissues and requires large amounts of DNA. Another nonviral approach involves the creation of liposomes, which are artificial lipid spheres with aqueous cores. These liposomes, which carry the therapeutic DNA, are capable of passing DNA through the target cell's membrane (Dzau et al., 1996; Niidome and Huang, 2002). Also, cationic polymers are used for gene delivery (Turunen et al., 1999). In comparison with viral vectors they provide superior control of their molecular composition, lower immunogenicity, flexibility of transgene size and commercial availability (Brown et al., 2001). The limitations of non-viral vectors are low transfection efficacy and transient gene expression.

Genes can be delivered into blood vessels in many ways. Systemic gene transfer means i.a. or i.v. injection of the gene vector. The advantage of systemic gene transfer is its easy gene delivery. Intravascular gene delivery can be done during angioplasty or stenting. Using i.a. gene transfer we can easily reach the target cells, including endothelial cells, macrophages, SMCs, T-cells and fibroblasts. However, anatomical barriers, which include internal elastic lamina, atherosclerotic lesions and blood complement system are the limitations of intravascular gene transfer because they inactivate many gene transfer vectors (Fechner et al., 1999;

Hiltunen et al., 2000b). Also, inflammatory and immunologic reactions cause problems. These limitations may be overcome by targeting vectors to certain cell types or tissues using antibodies, integrins or peptide libraries in order to discover feasible peptides to attach to the surface of the vector to achieve efficient gene transfer.

Several devices are available for intravascular gene transfer, including double balloon catheters (Breuss et al., 2002), dispath catheters (Hiltunen et al., 2000a) and porous and microporous catheters (Khang et al., 1996). NOGA catheters have been successfully used for intramyocardial gene transfer which also have the capacity for electrical mapping (Rutanen et al., 2004). The limitations of the current catheters include stopped blood flow and leakage through side branches.

Gene transfer can also be made *ex vivo* which requires the removal of a segment of the vein, cells or a specific organ. Gene transfer is then made *in vitro* and after that cells or the vein/organ segment can be transplanted back into the body (Kankkonen et al., 2004). Extravascular gene transfer can be done on the adventitial surface with a silastic or biodegradable collar or gel or by direct injection. In this way, extravascular gene transfer allows the vector to stay in close contact with the arterial wall for a long time. A common limitation is the difficulty in reaching the target cells i.e. endothelium and medial cells.

2.3.2. Re-targeting viral vectors

Most attempts to generate cell-targeting are based on receptor ligand interactions. Receptors are cell surface molecules which bind directly to their targets (ligands). Ligands such as transferrin and lipoproteins are endocytoced via receptor binding. After the ligand is bound, the receptors cluster and the endocytosis is mediated. Monoclonal antibodies or ligands (for example poly-lysin) for receptors have been used in receptor-mediated targeting (Wagner et al., 1992). The higher affinity of the antibody for the target corresponds to the higher targeting efficiency. Another strategy is to use peptides against proteins which are on the surface of the target cells. For example, by using peptide libraries, Koivunen et al have screened a selective gelatinase inhibitor (Koivunen et al., 1999) which targets specifically to MMP-2 and MMP-9. Furthermore, this cyclic peptide prevented tumour growth and invasion in animal models and improved the survival of mice bearing human tumours.

Although adenoviral vectors are the most efficient vectors for *in vivo* gene transfer used today, their transduction efficacy varies in different tissues (the basic structure of an adenovirus is presented in figure 3). The biodistribution studies show that intravascularily delivered adenoviral vectors spread systemically and lead

to the expression of transgenes in ectopic tissues (Hiltunen et al., 2000b). Also, problems with cytotoxicity and immune reactions are common. The cell entry of adenoviruses involves high-affinity binding of the viral fibre capsid protein to a cellular receptor CAR. Viral penton base binding to certain cellular integrins ($\alpha_v\beta_3$ and $\alpha_v\beta$) then mediates cell entry by receptor-mediated endocytosis via clathrin-coated pits. CAR receptors are expressed highly in hepatocytes and the myocardium whereas the endothelium and SMCs have low levels of CAR (Biermann et al., 2001). Re-targeting adenoviruses by altering virus tropism can be one way to solve these problems. However, precise optimisation and laborious studies for each disease application are needed since parameters relating to vector, tissue exposure time, route of delivery and target cell type, vary considerably.

There have been various strategies for improving the efficacy and cell specificity of re-targeted adenoviral vectors (figure 3). Mizuguchi and colleagues constructed CAR-binding ablated Ad vectors and alpha integrin-binding ablated Ad vectors by mutation in the FG loop of fibre knob and in the RGD motif of penton base (Mizuguchi et al., 2002). PEGylated adenoviruses (PEG-Ads) exhibit antibody evasion activity and long plasma half-life. However, their entry into cells has been prevented by steric hindrance by polyethylene glycol (PEG) chains. Eto et al PEGylated adenoviruses and combined an integrin targeted RGD motif on the tip of the PEG (Eto et al., 2004). They showed this modification could enhance gene expression in both CAR-positive and -negative cells. At the same time, these novel PEGylated adenoviruses maintained strong protective activity against antibodies.

Also, specific cell type targeted adenoviruses have been developed by combining fibre mutations that block CAR binding with genetic incorporation of SIGYPLP peptide (Nicklin et al., 2001). Further, there have been studies concerning the adenoviruses binding site modification using modifications that enhance binding to heparan sulphate receptors, inserting a receptor-binding motif RGD into the HI loop and at the C-terminus of the adenoviral fibre (Kibbe et al., 2000; Hay et al., 2001; Mizuguchi et al., 2002). A similar method was used when retargeting the adenoviral vector to other cellular receptors by inserting an arginine-glycine-aspartate (RGD) tripeptide in the fibre knob to treat the oesophageal carcinoma (Buskens et al., 2003).

Genetic modification of the adenovirus capsid is another strategy to achieve viral retargeting. Pereboeva et al presented a genetic fibre-mosaic virus, having two distinct fibres in one viral particle, as a means to facilitate fibre modifications allowing more flexibility in adenoviral retargeting approaches (Pereboeva et al., 2004). Work et al used dual targeting, combining a transductional targeting (see 2.3.3.) approach to improve vascular cell infectivity through RGD peptide insertion into adenovirus fibres, combined with transcriptional targeting to endothelial cells using an approximately 1 kb fragment of the fms-like tyrosine kinase receptor-1 (FLT-1)

promoter. They showed that double modification shifted transduction profiles towards vascular endothelial cells (Work et al., 2004).

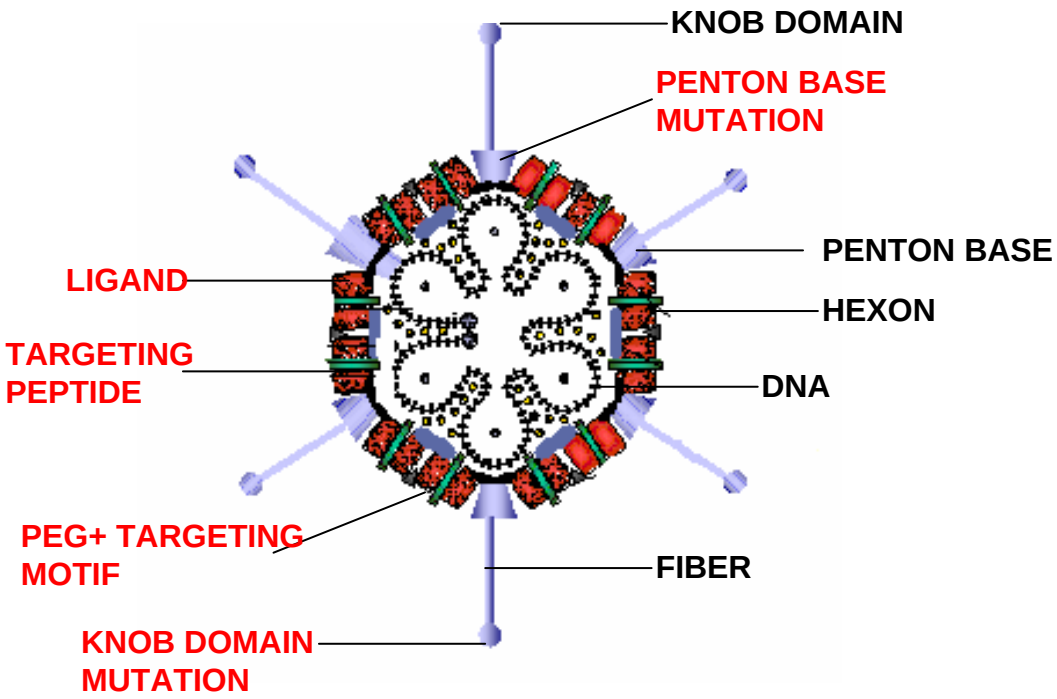

Figure 3. The structure of an adenovirus. The basic structure is indicated using black letters. The different ways to retarget adenoviruses are indicated using red letters.

2.3.3. Targeting of transgene expression

The previous section mainly dealt with vector-cell interaction. However, the targeting of vectors can also be made at a transcriptional level using tissue specific promoters. Various studies have been done using different promoters. Nicklin et al constructed an EC specific promoter and studied its efficacy in vitro and in vivo (Nicklin et al., 2001). The results were promising, suggesting enhanced gene expression. Gruchala et al presented opposite results in AAVs. They used an EC-specific Tie-1 promoter. It is known that the EC specific receptor Tie-1 is up-regulated by disturbed flow in atherogenic vascular niches. Studies showed that the use of a specific promoter did not lead to specific transgene expression in EC (Gruchala et al., 2004). Also, SMC specific promoters for adenoviral gene therapy have been developed (Akyurek et al., 2000) with low expression levels. Further, Appleby et al showed that by combining an SMC specific promoter and enhancer a 40-fold increace in transduction efficacy was achieved in vivo and a 90-fold increase in vitro (Appleby et al., 2003). Ebara and colleagues tested the properties of promoters on adenoviral vectors. Their studies suggest that promoter selection can also influence the toxic effects of an adenoviral gene therapy vector (Ebara et al.,

2002). To sum up, mainly positive results have been achieved using tissue specific promoters. However, more studies are needed to evaluate their efficacy in vivo.

Regulated expression of therapeutic genes is required for long-term gene therapy applications for many disorders. Regulated gene delivery systems are usually made of two elements: an inducible promoter and a transactivator. A Tc-dependent transcriptional switch has been used for regulation. Doxicyclin acts as an inducer drug. Doxicyclin binds to the repressor protein and induces the release of the repressor. The most studied regulator systems are TetON3 and Tet OFF2. There are a few studies suggesting that regulated gene therapy gives siqnificant benefits for long term gene expression (Koponen et al., 2003; Qu et al., 2004).

2.3.4. Treatment genes

Tissue inhibitor of metalloproteinase (TIMP)

Physiological and pathological vascular remodelling requires the reorganization and degradation of ECM in the vessel wall. The enzymes which participate and are specialized for this task are called matrix metalloproteinase's (MMPs). MMPs are a family of enzymes with the ability to degrade ECM components. Lasting change in blood vessel structure demands remodelling of its matrix scaffold. MMPs contribution has been studied in relation to pathological vascular conditions characterized by wall remodelling such as atherosclerosis, the development of restenotic lesions, the failure of the vein grafts and atherosclerotic plaque disruption. It has been found that in diseased human arteries there is an increased expression of basement membrane-degrading metalloproteinase's (MMP-2 and MMP-9) (Dollery et al., 1995; Nikkari et al., 1996). In normal vascular tissues, endothelial cells, medial SMCs and adventitial connective tissue cells are the main sources of MMP-2 (gelatinase A) and MMP-9 (gelatinase B). However, macrophages and other types of infiltrating cells are known to be important sources of MMPs in a wide variety of inflammatory conditions.

MMP activity is regulated at three levels: gene expression, activation of proenzyme forms of MMPs, and inhibition by complexing with their specific TIMPs (Nagase, 1997). The expression of MMPs is also controlled at the transcriptional level by cytokines, hormones and growth factors and MMP activity in macrophages is upregulated by nitric oxide, immunologic activation and reactive oxygen species. Currently, four different TIMPs are known. TIMP-1, TIMP-2 and TIMP-3 are found in vascular tissue (George, 1998; Brew et al., 2000). TIMP-4 is expressed predominantly in the heart (Leco et al., 1997). The balance between MMPs and TIMPs affects ECM stability and an excess of MMPs is likely to degrade the ECM. TIMPs act on two levels: they bind

to the carboxy terminus of proenzyme forms of MMPs to retard MMP activation, and they bind activated MMPs at the active site of the enzyme. TIMP-1 has been found to act as an endogenous inhibitor of MMP-2 and MMP-9 (Dollery et al., 1995; George, 1998).

A strong local MMP over-expression and *in situ* matrix-degrading activity is present, especially in vulnerable shoulders of human atheroma. Also, inhibition by ubiquitously expressed TIMPs has been found in the areas subjected to the highest mechanical stress. These findings have provided a potential mechanistic insight into the process of plaque destabilization through matrix weakening by MMPs (Galis and Khatri, 2002). Analysis of human coronary atherectomy specimens revealed active synthesis of MMP-9 by macrophages and SMCs in lesions of patients with unstable versus stable angina, suggesting the role of MMP-9 in acute syndromes (Brown et al., 1995).

Peripheral blood levels of MMP-2 and MMP-9 may be increased in patients with acute coronary syndrome, raising an interesting question of the possibility of developing non-invasive tests for the detection of plaque vulnerability (Kai et al., 1998). Ever-increasing data illustrates the key role of MMPs in many of the processes that control vascular remodelling and especially the formation and progression of atherosclerotic plaques. The net effect of the various triggers shown to increase MMP activity in the setting of atherosclerosis and vascular remodelling is an imbalance of the MMP:TIMP ratio in favour of ECM degradation. Thus, it is sensible that modulation of MMP activity or the MMP:TIMP balance may be useful in the treatment and prevention of atherosclerosis.

There have been a variety of studies concerning the effect of TIMPs gene therapy on restenosis and vein graft stenosis. Over expression of TIMP-1 or -2 inhibits neointima formation in the rat model of angioplasty restenosis and *in vitro* human model of vein graft neointima formation (Forough et al., 1996; George et al., 1998a; George et al., 1998b; Dollery et al., 1999). AAV mediated TIMP-1 gene transfer inhibited neointima formation lasting up to several months in a rat model (Ramirez Correa et al., 2004). Also, suppressing SMC migration and proliferation by inhibiting MMPs, TIMP-3 has been shown to inhibit neointima formation in human and porcine models (George et al., 2000). Furthermore, Adenovirus mediated TIMP-4 gene transfer has been shown to inhibit SMC migration and induce apoptosis in vitro and in vivo (Guo et al., 2004). Thus, TIMPs are an interesting group of enzymes due their involvement in the pathogenesis of coronary diseases. However, further studies are needed to evaluate their efficacy in clinical studies.

Vascular endothelial growth factors (VEGFs)

Vascular endothelial growth factors (VEGFs) are regulators of angiogenesis and they stimulate endothelial cell proliferation, increase endothelial permeability and act as a survival factor in retinal vessels (Ferrara and Bunting, 1996). Angiogenesis takes place in several pathological conditions such as diabetic retinopathy, tumour growth and rheumatoid arthritis (Folkman, 1995). Therefore, administration of the gene for VEGF is a useful tool for providing both angiogenesis and the stimulation of endothelial repair. Both mechanisms are useful for the treatment of restenosis and vein graft stenosis.

VEGFs are secreted, highly conserved dimeric proteins which are active as an endothelial cell specific mitogen (Murohara et al., 1998; Senger et al., 1993). Five members in the human VEGF-family have been identified: VEGF -A, -B, -C, -D and PlGF (placental growth factor) differing in their ability to bind to three VEGF receptors (Figure 4 presents the VEGFs and their binding to the receptors) (Achen et al., 1998; Maglione et al., 1991; Joukov et al., 1996). The signalling is mediated by three receptor tyrosine kinases (VEGF-R1, VEGF-R2 and VEGF-R3) (Korpelainen and Alitalo, 1998). VEGF receptors are expressed in various cell types but ECs and endothelial progenitor cells are the primary targets for VEGFs (Ferrara et al., 2003). Recently, two co-receptors have been identified. Neuronal guidance receptors neuropils NRP-1 and NRP-2 are required for normal embryonic blood and lymphatic vessel development. NRPs seem to amplify signal transduction mediated by VEGFRs (Murga et al., 2004; Oh et al., 2002).

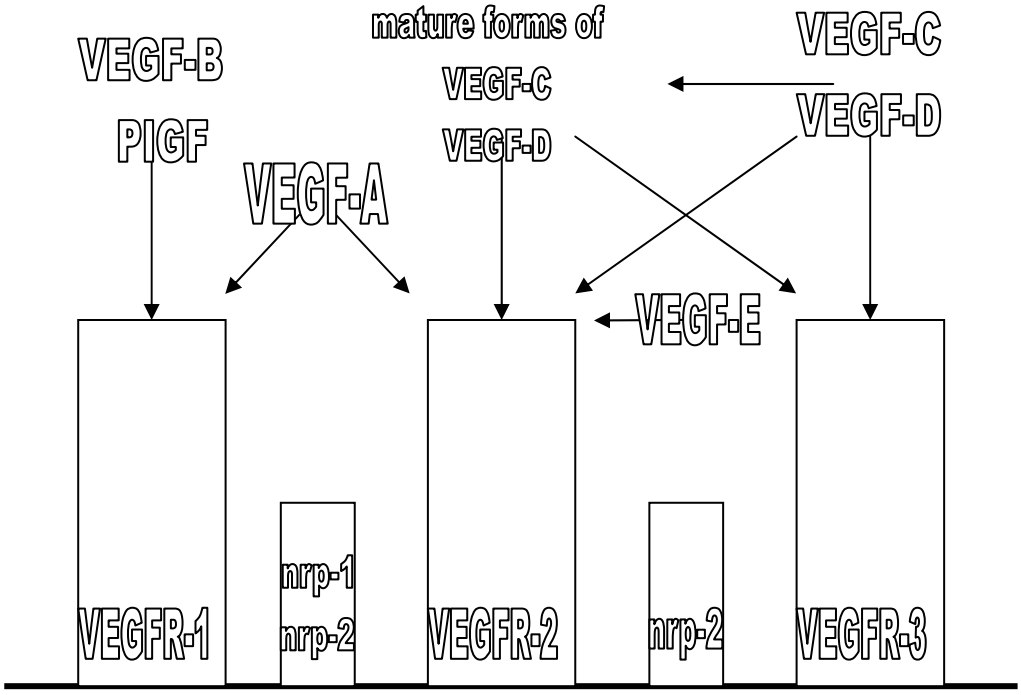

Figure 4. The signalling of VEGFs via their receptors (VEGFRs).

The VEGF-A gene is a relative of platelet-derived growth factor (PDGF) and five different isoforms are generated by alternative splicing from a single VEGF gene, distinguished by their heparan and heparin sulphate binding properties (Poltorak et al., 1997; Leung et al., 1989; Ferrara, 1999). VEGF-A is induced by hypoxia, hypoglycaemia, inflammation, tissue repair and malignancy. VEGFR-1, VEGFR-2 and the production of nitric oxide (by eNOS and iNOS) mediates actions of VEGF-A including vasodilatation, vascular permeability and angiogenesis (Laitinen et al., 1997; Murohara et al., 1998). It also plays a great role in the deposition of ascites fluid, extra vascular fibrin, tissue oedema in tissue repair, inflammation and cancer (Dvorak et al., 1995). It has also been suggested that proteases such as plasmin and MMPs are up-regulated by VEGF-A (Wang and Keiser, 1998).

VEGF-B has been shown to stimulate the growth of human and bovine vascular endothelial cells (Olofsson et al., 1996). At least two variants of spliced VEGF-B are expressed. VEGF-B has been shown to be an endothelial cell mitogen in vitro and to regulate plasminogen activity in ECs. It is primarily distributed in the skeletal muscle and myocardium and it is co-expresed with VEGF-A, which can also form heterodimers. VEGF-B is expressed as a membrane-bound protein that can be released in a soluble form after the addition of heparin (Olofsson et al., 1996). VEGF-B binds to VEGFR-1 whereas heterodiomers can also bind to VEGFR-2.

VEGF-C and VEGF-D are a subfamily of VEGF because of their similaritiy in being 48% identical in structure. Both enclose C- and N-terminal extensions and have a similar receptor binding profile. They are also synthesized as precursor forms which are proteolytically processed into mature forms (Achen et al., 1998; Stacker et al., 1999). Both promote tumour angiogenesis, lymph angiogenesis and metastatic spread. The full length forms of VEGF-C and VEGF-D use VEGFR-3 as signalling whereas the mature forms use VEGFR-2 (Joukov et al., 1996; Stacker et al., 1999). The full length forms of VEGF-C and –D are mainly lymphangiogenic whereas short mature forms are angiogenic and promote permeability in vessels (Rissanen et al., 2003).

VEGF-E is a viral homologue of VEGF and is encoded by the Orf virus. VEGF-E binds selectively to VEGFR-2. Angiogenesis and vascular permeability are promoted by the Orf virus skin infection and by recombinant VEGF-E (Wise et al., 1999). PIGF is expressed in the placenta, lung, thyroid and goitre. It has 53% homology with a PDGF-like region of VEGF and it binds to VEGFR-1. The biology of PIGF is not yet fully understood but

is has been shown to promote monocyte and haematopoietic stem cell recruitment from the bone marrow. It is believed that PIGF may up-regulate VEGF expression (Bottomley et al., 2000).

Studies have been made concerning the effects of VEGF gene therapy on restenosis. The primary aim has been to achieve rapid re-endothelialization because intravascular manipulations damage the endothelial cells. It has been demonstrated that both VEGF-A and VEGF-C gene therapy reduced restenosis after arterial injury (Hiltunen et al., 2000a). The problem with delayed reendothelialization and late thrombosis using drug-eluting stents was studied in a rabbit model using plasmid VEGF eluting stents. Walter et al found that the reendothelialization was accelerated using VEGF eluting stents and suggested the use of VEGF eluting stents as a stand-alone or as combination therapy (Walter et al., 2004). However, controversial data has also been published, suggesting that VEGF eluting stents do not accelerate reendothelialization or inhibit neointima formation. Only stent thrombosis was reduced (Swanson et al., 2003). In one study, VEGF-D gene transfer inhibited neointimal growth in rabbits via NO-dependant mechanism (Rutanen et al., 2002b). Recently, it has been reported that intramyocardial gene transfer using the mature form VEGF-D induced transmural angiogenesis in a porcine heart (Rutanen et al., 2004).

In a clinical trial intravascular VEGF-A plasmid/liposome gene transfer was done to human coronaries at the same time as PTCA in a phase II study (Hedman et al., 2003). It was found that intracoronary gene transfer can be performed safely and no differences in clinical restenosis were present after the 6-month follow-up. A significant increase was detected in myocardial perfusion with the adenovirus mediated, VEGF treated patients. In a randomized, controlled phase II study, a catheter mediated VEGF-A gene transfer was done using a plasmid/liposome or an adenovirus. When a gene was transferred to infrainquinal arteries after PTA, increased vessel formation was found in the VEGF treatment group, but no effect on restenosis was detected (Makinen et al., 2002).

It is concluded that adenovirus medited VEGF-C and –D gene transfers are efficient at decreasing restenosis in rabbit models and are attractive targets for clinical studies.

Platelet activating factor acetylhydrolase

Platelet activating factor (PAF) is a phospholipids autacoid. PAF triggers neutrophil binding to the endothelial cells and causes rapid extravasation of leukocytes and monocytes (Imaizumi et al., 1995). PAF has many functions: It stimulates monocyte secretion of cytokines and SMC growth but it also causes vasodilatation,

platelet aggregation, anaphylactic shock and increased vascular permeability. Having inflammatory mediator functions, PAF activates platelets, neutrophils, monocytes, vascular SMCs and macrophages (Prescott et al., 2000). PAF is being produced by monocyte-macrophages, neutrophils, platelets, mast cells and endothelial cells. PAF is retained on the EC surfaces and is involved in the early steps of inflammation. Platelet activating factor acetylhydrolase (PAF-AH) is an enzyme capable of hydrolyzing platelet-activating factor (PAF).

PAF-AHs are structurally diverse isoenzymes which catalyze the hydrolysis of the alkyl group of glycerol in bioactive phospholipids. PAF-AH inactivates PAF and PAF-like oxidized phospholipids by hydrolyzing phospholipids with shortened or/and oxidized sn-2 residues. PAF-AH converts PAF to lysoPAF in a reaction that does not require calcium. Macrophages, mast cells and hepatocytes synthesize secreted PAF-AH. However, in the plasma PAF-AH originates from haematopoietic cells and it's linked to the lipoproteins, mainly to LDL. PAF-AH is thought to have a protective role and to prevent LDL oxidation. During oxidation PAF-AH activity is decreased and is rapidly inactivated by oxygen radicals (Stafforini et al., 1992).

Endothelial cells synthesize PAF-AH locally during thrombotic events. ECs also produce PAF in response to oxidative stress. In addition, thrombin, bradykinin and histamine can induce monocytes to secrete PAF. PAF-AH is also found to be expressed by macrophages in human and rabbit atherosclerotic lesions (Häkkinen et al., 1999). These facts suggest that PAF may contribute to thrombotic events and the development of atherosclerosis. It has been shown that in the plasma, PAF-AH activity is lower after acute myocardial infarction. Also, it has been shown that the gene transfer of human PAF-AH prevents injury-induced neointima formation and reduces spontaneous atherosclerosis in apolipoprotein E-deficient mice (Quarck et al., 2001). Turunen et al recently showed that the adenovirus-mediated gene transfer of PAF-AH reduces LDL degradation and foam cell formation in vitro (Turunen et al., 2004). It has also been shown that PAF-AH in plasma, is increased in patients with atherosclerotic diseases (Satoh et al., 1992). Packard et al showed that elevated levels of PAF-AH appear to be a strong risk factor for coronary heart disease (Packard et al., 2000). However, the PAF-AH enrichment may be of pathophysiological relevance in preventing the oxidation of LDL preceeding any further (Kovanen and Pentikainen, 2003). Thus, the high levels of PAF-AH would indicate the overall risk of coronary heart disease and not be a risk factor in itself.

35K

Chemokines are small heparin binding proteins that are classified into C, CC, CXC or CX_3C subfamilies (X = amino acid). The biological activities of chemokines are mediated through interactions with transmembrane

receptors expressed by target cells. C chemokines (lymphotactin) attract T and NK cells. CC chemokines attract macrophages and T cell populations and CXC chemokines attract neutrophils. CX_3C chemokine (fractalkine) attracts monocytes, T cell subsets and NK cells. Over 50 chemokines and 16 chemokine receptors have been described (Rossi and Zlotnik, 2000). Chemokines play a critical role in the host response to viral infections. Thus, several viruses have evolved strategies to interfere with the functioning of chemokines. For example, poxviruses and vaccinia viruses express secreted proteins, which bind to human and rodent chemokines with great affinity and competitively inhibit their interaction with cellular receptors (Alcami et al., 1998; Graham et al., 1997). One of their main functions is to direct the migration of immune cells to sites of inflammation and infection.

The CC-chemokine group is the largest subfamily of chemokines and they have been found to be important factors in the pathogenesis of rheumatoid arthritis, asthma, multiple sclerosis, transplant rejection and atherosclerosis. The vaccinia virus expresses a 35 kDa protein (35K) which binds with high affinity to nearly all of the chemokine CC-class but not to the other classes (Burns et al., 2002). *In vitro* studies show that 35K inhibits chemokine signal transduction and cell migration (Bursill et al., 2003). As a result, it may be used to block CC-chemokine-induced monocyte/macrophage recruitment and several inflammatory factors associated with the progression of restenosis and vein graft stenosis. The mechanism of CC-chemokine inhibition by 35K is not known, but it has been suggested that 35K binds the CC-chemokines, sequesters them, prevents them from binding to the endothelium and thereby prevents them from exerting their function (Bursill et al., 2003). Genetic evidence for a role of chemokines and their receptors in human population studies remains under investigation. Recently, the same group showed that a single intravenous injection of a recombinant adenovirus encoding the broad-spectrum CC inhibitor 35K can reduce atherosclerosis by inhibiting CC-induced macrophage recruitment in atherosclerotic ApoE KO mice. The result suggested that CCs are important factors in atherogenesis (Bursill et al., 2004).

2.3.5. Combination gene therapy

The majority of the studies of restenosis and vein graft stenosis involving gene therapy, have usually been based on a single treatment gene. When we have learnt the pathophysiology of the diseases better and noticed their complex events, it may be that combination gene therapy (gene cocktails), can provide a better treatment effect. It may be that using treatment genes with different mechanisms known to reduce restenosis we might end up with an additive treatment effect.

The present combination gene therapy studies have primarily concerned cancer gene therapy where beneficial additive effects have been noticed. For example, the inhibition of angiogenesis using adenoviral-mediated endostatin gene transfer together with the HSV-tymidine kinase gene therapy, resulted in an improved treatment effect in renal cell cancer compared to the single gene treatments (Pulkkanen et al., 2002). It has also been shown that adenovirus-mediated gene therapy using the Herpes simplex virus thymidine/ganciclovir system and murine interleukin-12 induced effective additive antitumour activity against medullary thyroid carcinoma (Yamazaki et al., 2004). Also, Tai et al showed that concurrent delivery of GM-CSF and endostatin genes by a single adenoviral vector provides a synergistic effect on the treatment of orthotropic liver tumours (Tai et al., 2003). Effective treatment results were also noticed with synergistic combinations of tumour-suppressing gene therapy such as Ad-FHIT and Ad-p53 for non-small cell lung cancer (Nishizaki et al., 2004).

So far, only a few studies have been published on combination gene therapy and its effects on the vascular remodelling processes. It has been shown that a combination of intravascular VEGF-C gene transfer and treatment with PDGF receptor kinase inhibitor STI571, leads to a long-term reduction in neointima formation in balloon-denuded rabbit aortas (Leppanen et al., 2004). This study showed a prolonged therapeutic effect when compared to a single treatment with VEGF-C or STI571 (Leppanen et al., 2000; Hiltunen et al., 2000a). It has also been shown that brief irrigation with tissue factor pathway inhibitor (TFPI) combined with adenovirus-mediated local TFPI gene transfer, reduced neointima formation in rabbits (Atsuchi et al., 2001).

3. AIMS OF THIS STUDY

The aim of this thesis was to study the gene therapy for restenosis and vein graft stenosis using different treatment methods. The following questions were addressed:

1. Does re-targeting adenoviruses to MMPs have a better treatment effect on restenosis when compared with non-targeted adenoviruses? Moreover, does vector targeting alter tropism (STUDY 1)?

2. Is single gene therapy sufficient for the treatment of restenosis, or is two gene combination therapy needed to achieve more efficient treatment results in reducing neointima formation (STUDY 2)?

3. Does antioxidant gene PAF-AH have the effect of reducing restenosis in a rabbit model (STUDY 3)?

4. Can the anti-inflammatory protein 35K reduce stenosis in a rabbit vein graft model and is it more effective than TIMP-1 gene transfer (STUDY 4)?

4. MATERIALS AND METHODS

4.1. Gene transfer vectors (I-IV)

TIMP-1, VEGF-C, 35K and lacZ adenoviruses used for the study have been described previously (Laitinen et al., 1998; George et al., 1998b; Bursill et al., 2003; Hiltunen et al., 2000b). Replication-deficient E1-partial E3 deleted adenoviruses were produced in 293 cells (Laitinen et al., 1998). The helper-free virus was amplified using three separate rounds of plaque lysis and purified and concentrated by ultracentrifugation. Titer assay, Southern blotting, E1/E2 selective PCR analysis and cytopathic effect assay on A549 cells were used for the final characterization of the viruses (Puumalainen et al., 1998). All viral lots were analyzed for the absence of microbiological contaminants, mycoplasma and lipopolysaccharide (Puumalainen et al., 1998). The TIMP-1, VEGF-C, 35K and lacZ adenoviruses were driven by the CMV promoter and the lacZ adenovirus also had a nuclear targeted signal.

The PAF-AH adenoviruses were constructed and produced using the Adeno-X™ Expression system. Human PAF-AH cDNA was cloned into ADENO-X-Viral DNA using the CMV immidiate early promoter and bovine growth hormone polyA. Fugene 6 was used for plasmid transfection and viruses were produced in the HEK 293 cell line.

4.2. Re-targeting (I)

The chimeric peptides containing poly-Lys (K16) with MMP-2/MMP-9 targeting peptide (KKKKKKKKKKKKKKKKGCTTHWGFTLCGS) and the control peptide (KKKKKKKKKKKKKKKKGCFWTLTGTHCGS) were synthesized using the PerSeptive 9050 Plus automated peptide synthesizer with Fmoc strategy. For resin, the NovaSyn TGA with 4-hydroxymethylphenoxyacetic acid linker with pre-coupled Cys(trt) was used. The peptides were purified by HPLC (Shimadzu, Japan) with C18 reverse phase column and acetonitrile as eluent (0.1%TFA in H2O/0-60% acetonitrile gradient for 60 min) and verified with a MALDI-TOF mass spectrometer (Bruker, Germany). Ten milligrams of each peptide were cyclized using the Iodination method. Cyclized peptides were purified with HPLC as described above and the formation of the sulphur-bridge was verified by mass spectrometry. The cyclic peptide was attached to the poly-lysine chain (n=16) using 2 glycine spacers to allow more flexibility for the cyclic residue. The peptide was attached to the adenovirus surface by a transglutaminase reaction, which makes covalent bonds between lysine-residues of the peptide and glutamine residues of the adenovirus surface (Wagner et al., 1992).

4.3. *In vitro* studies (I)

ONPG assay was used to study the re-targeted adenovirus' *in vitro* efficacy. Modified adenoviruses and a control adenovirus coding for lacZ were produced as described above. Virus concentrations were measured using spectrophotometry at 260nm. Multiplicity (MOI; viral particles per cell) of infection 0.1 and 1 were used in cell culture studies. Several virus complexes were made for the experiments: The first virus complex had one lysine residue on the surface (AdlacZ(lys)), the second complex had poly-lysine (AdlacZ(p-lys), the third complex had HWGF-targeted peptide poly-lysine (AdlacZ(HWGF)) and the fourth complex had the mismatch peptide poly-lysine on the surface (AdlacZ(MM)). β-galactosidase activity in the rabbit aortic smooth muscle (RAASMC), the endothelial tumour cell hybrid (EaHy) and the hepatocyte (HEP-G2) (HB-8065) cell lines were measured after transduction at MOI of 1 and 0.1 for four hours. O-nitrophenyl β-D-galactopyranoside (ONPG) was used as a colorimetric substrate as described.

4.4. Animal studies

4.4.1. Restenosis model (I-III)

The rabbits were kept on a 0.25% cholesterol diet starting two weeks prior to operation. The number of animals was from five to seven in each treatment group. Intimal proliferation was induced by endothelial denudation. The whole aorta was denuded twice using a 4.0F embolectomy catheter (Sorin Biomedical). After three days of denudation a gene transfer was performed. A 3.0F channelled balloon drug delivery catheter (Dispatch, Boston Scientific) was introduced into the abdominal aorta via a 5F introducer sheath which was in the right carotid artery. The catheter was introduced under fluoroscopical control. The position of the gene transfer in the aortas was selected using fluoroscopical control to avoid the possible leakage of side branches during the gene transfer. The anatomical location of the catheter was determined measuring its distance from iliac bifurcation. The gene transfer was performed over 10 minutes with 6ATM pressure.

4.4.2. Vein graft model (IV)

The animals numbered from five to seven in each treatment group. End to side anastomosis was performed on the right common carotid artery using the right external jugular vein. The vein was dissected free from the surrounding tissues and the side branches were legated. The proximal side of the vein was clamped and the

bifurcation branches were legated. A puncture was made in the right bifurcation vein right behind the legation knot. Through the hole a cannula was inserted into the vein and the cannula was fixed with a knot. The vein was flushed with saline before the injection of the virus. The virus was kept in the vein for 10 minutes with slight pressure (figure 5). No virus was released into systemic circulation. The virus was removed and the vein was detached below the bifurcation. The carotid artery was dissected free from the tissue and clamped at two places to stop the blood flow. A hole was made in the artery and the free end of the vein was connected to the carotid artery using 8.0 Dexon knots. Clipses were removed and the blood flow was established (figure 5).

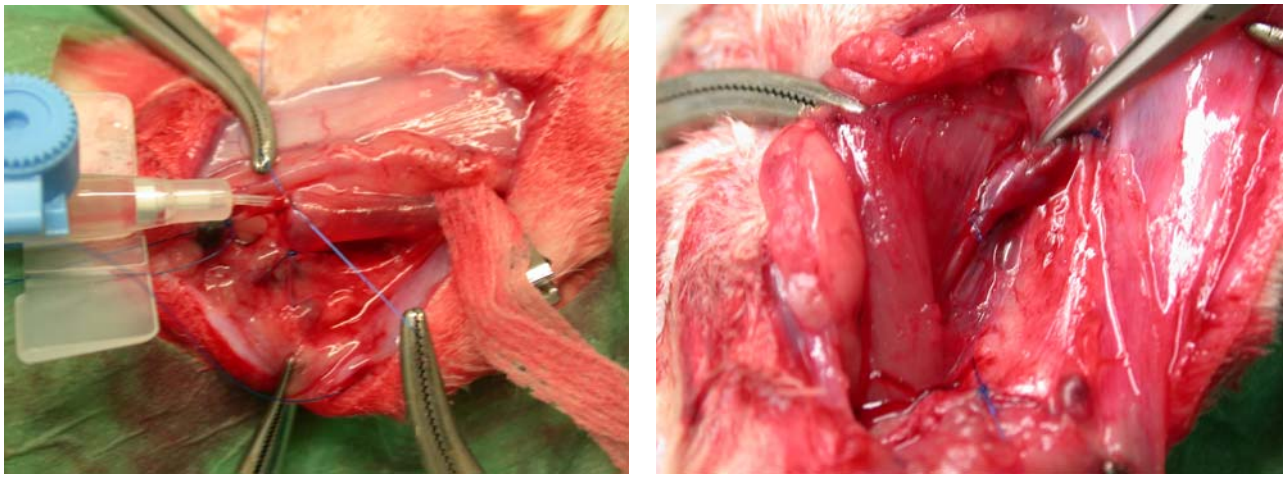

Figure 5. Vein graft model. Ex vivo gene transfer is performed in the first picture. The second picture shows the anastomosis and the blood flow.

4.5. Histological analyses (I-IV)

After sacrifice, the transduced segment and the organ samples (liver, spleen, lungs, gonads, kidney) were removed for biodistribution analyses. In the restenosis studies a control segment of aorta was also removed. The transduced and the proximal control segments were divided into six equal parts. The most proximal and the most distal parts were snap frozen in liquid nitrogen and stored at -70 °C. The next proximal and the next distal segments were immersion-fixed in 4% paraformaldehyde/15% sucrose for 4h, rinsed with 15% sucrose overnight and embedded in paraffin. The two middle segments were immersion fixed in 4% parformaldehyde/phosphate buffered saline for 10 min, embedded in OCT-compound and stored at -70 °C.

Biotinylated versions of the HWGF and MM peptide / poly-lysine complexes were constructed and tested on placental tissue sections since the placenta is known to produce high levels of MMPs. Briefly, paraffin-embedded sections of a New Zealand White rabbit placenta were deparaffinized and pretreated for 25 min with 1% TritonX-100, and were then boiled for 10 min in 0.01M of sodium citrate. Slides were blocked for 20

min with a CAS blocking solution (ZYMED) and then incubated for 60 min with biotinylated peptide complexes (1:100), followed by 30 min of incubation with Oregon Green Neutravidin (1:100).

Immunohistochemical stainings were performed (table 3) using various antibodies.

specificity	antibody	dilution
macrophages	RAM-11	1:50
endothelium	CD31	1:50
proliferating cells	BrdU	1:100
SMC	HHF35	1:50
T-cells	MCA 805	1:100
TIMP-1	clone IM63	1:10
MMP-2	clone IM33L	1:25
MMP-9	clone IM37L	1:25
apoptosis	Apotag kit (Intergren)	(Pulkkanen et al., 2002)

Table 3. Immunohistochemical stainings performed and dilutions used.

Control immunostainings were conducted without primary antibodies and with class and species-matching primary antibodies. Morphometry and analysis of I/M ratios were performed using an Olympus AX70 microscope and analysis software (Soft imaging systems, GmbH).

Gene transfer efficacy was evaluated using X-gal staining of OCT-embedded tissue sections for 12 hours (Puumalainen et al., 1998).

4.6. RT-PCR (I-IV) and quantitative RT-PCR (TaqMan) (I)

For the assessments of mRNA expression of transduced genes RT-PCR and quantitative RT-PCR (TaqMan) was performed with the following primers (table 4).

The TIMP-1 expression level in AdTIMP-1 and AdTIMP-1(HWGF) transduced liver samples were analyzed with quantitative TaqMan RT-PCR. Total RNA was extracted, DNAse treated and reverse transcribed as for RT-PCR. An aliquot of cDNA was amplified with primers (table 4). A TaqMan probe containing the fluorescent reporter dye 6-carboxyfluorescein (FAM), was covalently linked to the 5' end of the oligonucleotide and a quencher dye 6-carboxytetramethylrhodamine (TAMRA) attached to the 3' end via a linker group (PE Biosystems, Foster City, CA). To construct a standard curve, dilutions of cDNA obtained from AdTIMP-1

transduced RAASMC cells were amplified. The reaction mixture consisted of 1×Universal Master Mix (PE Biosystems), 300 nM each primer and 100 nM probe. Samples (standards, unknowns, no template controls and no reverse transcription controls) were amplified for 40 cycles in a PE Biosystems 7700 sequence detection system. The data was processed by the SDS 1.6 software (PE Biosystems) to generate standard curves and to determine the concentration of the target in unknown samples by interpolation. Input amounts of cDNAs were corrected by amplification of the 18S ribosomal RNA as an endogenous control using TaqMan Ribosomal RNA Control Reagents (PE Biosystems). Expression levels from triplicate liver samples (for AdTIMP-1 n=3 and for AdTIMP-1(HWGF) n=5) were averaged and normalized to 18S RNA expression.

TIMP-1 (500bp) forward	5'-ACCCAACGACGGCCTTCTGCAATTC-3'
TIMP-1 reverse	5'-GGCTATCTGGGACCGCAGGGACTGC-3'
VEGF-C (285pb) forward	5'-CCCACCAATTACATGTGGAA-3'
VEGF-C reverse	5'-GTTGATTTCTGGGGCAGGTT-3'
35K (243bp) forward	5'-ATCCTCATCCTCCTCCTCGT-3'
35K reverse	5'-CTCAGACCTCCACCGATGAT-3'
lacZ forward	5'-TGAGGGGACGACGACAGTAT-3'
lacZ reverse	5'-TTGGAGGCCTAGGCTTTTGC-3'
lacZ nested (218bp)forward	5'-GGTAGAAGACCCCAAGGACTTT-3'
lacZ nested reverse	5'-CGCCATTCGCCATTCAG-3'
Lp-PLA_2 forward	5'-TGGAGCAACGGTTATTCAG-3'
Lp-PLA_2 reverse	5'-TGGTTGTGTTAATGTTGGTCC-3'
TIMP-1 (TaqMan) forward	5'-GACGGCCTTCTGCAATTCC-3'
TIMP-1 (TaqMan) reverse	5'-GGTGGTCTGGTTGACTTCTGGT-3'
TIMP-1 (TaqMan) probe	5'-ACCTCGTCAGGGCCAAGTTCGT-3'

Table 4. Primers used in RT-PCR and quantitative RT-PCR studies.

The RT-PCR for VEGF-A was made according to (Hiltunen et al., 2000a).

4.7. Statistical analysis (I-IV)

The statistical analysis was performed using ANOVA, followed by a modified Student's t-test. Additionally, the Chi-square test was used. For data processing Microsoft Excel and SPSS software were used. Results were considered significant at $p < 0.05$.

5. RESULTS

5.1. Gene therapy for restenosis in a rabbit model (I-III)

Efficacy of a re-targeted adenoviral vector in vitro and in vivo (I)

In this study an MMP-2 and MMP-9 targeted adenovirus was made. Koivunen et al (Koivunen et al., 1999) had developed, by using peptide libraries, a selective gelatinase inhibitor. That cyclic peptide (HWGF) was linked via a poly-lysine spacer (Wagner et al., 1992) to the surface of the adenovirus. Since the placenta has high MMP production, the binding properties of the MMP-2 and MMP-9 targeted (HWGF) peptide were tested using rabbit placental histological. Fluorescing labelled HWGF peptide, was detected microscopically on the trophoblast cells (figure 6). Mismatch peptide showed only low background fluorescence. We concluded that the HWGF sequence targets MMP-2 and MMP-9 expressing cells in rabbit tissue.

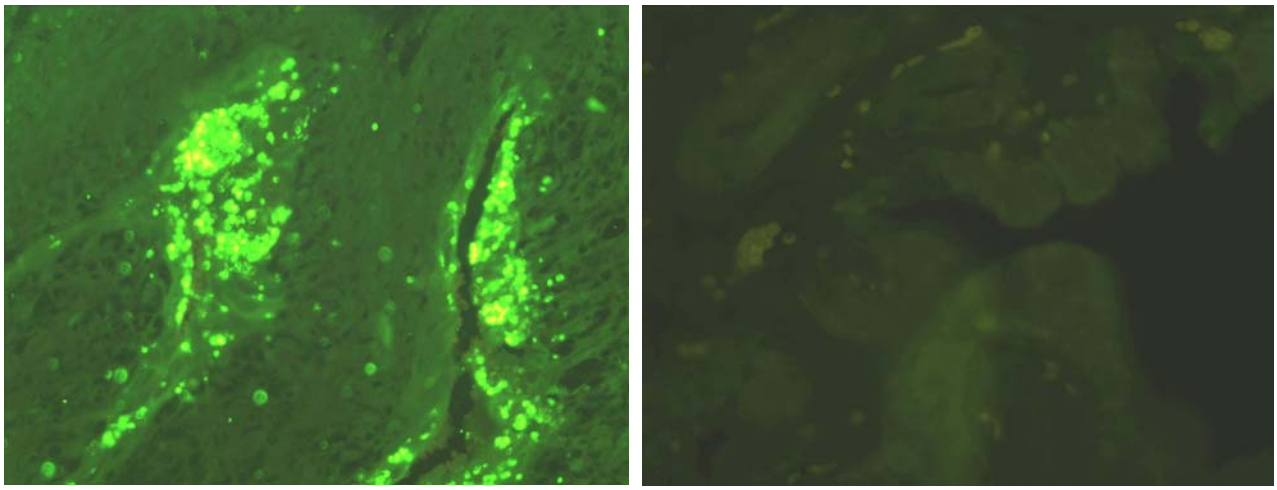

Figure 6. MMP-targeting peptide on placental sample in the left picture, mismatch peptide on placental sample in the right picture. The MMP-positive trophoblasts are cells expressing fluorescence.

I*n vitro* transfection efficacy using ONPG assay has been studied (Puumalainen et al., 1998). β-galactosidase activity was quantified after the transduction of RAASMC, EaHy and Hep-G2 cells. 0.1 and 1 multiplicity of infection (MOI) was used and unmodified AdlacZ was used as a control. In RAASMCs AdlacZ(HWGF) showed an 8-fold increase in transduction efficacy when compared with AdlacZ. The mismatch peptide-modified adenovirus (AdlacZ(MM)) mediated a 2-fold increase in transduction efficacy. In endothelial cells β-galactosidase expression levels were 12.5-fold higher with AdlacZ(HWGF) than with the control virus. In contrast, in the Hep-G2 cells, AdlacZ(HWGF) mediated significantly lower transduction than the control. Also, AdlacZ(MM) mediated lower transduction efficacy than the control. In addition, lysine modified adenoviruses (AdlacZ(lys)) tended to decrease the transduction efficacy.

In animal studies the endothelial damage was caused by angioplasty. At a two week time point the AdlacZ and AdlacZ(HWGF) control groups had the highest intima/media (I/M) ratios whereas AdTIMP-1(HWGF), AdTIMP-1(MM) and AdTIMP-1 showed decreased intimal thickening. The difference in I/M ratio between AdlacZ(HWGF) and the AdTIMP-1(HWGF) group was significant ($p<0.05$) at the two week time point. As shown previously, the AdTIMP-1 gene transfer also tended to reduce intimal thickening compared to the control groups (Forough et al., 1996). No increase in inflammation or macrophage accumulation was detected in the histological stainings.

By using RT-PCR specific to the transduced TIMP-1 gene, mRNA expression was detected two weeks after the gene transfer in the aortas, which was also shown by immunostainings. Expression of the modified and intact AdlacZs in the liver was analyzed using the X-Gal staining method (Hiltunen et al., 2000b). Rabbits that had had either AdlacZ(HWGF) or AdlacZ(MM) gene transfer showed no X-Gal positive cells in the liver whereas positive cells could be seen in the liver after the control AdlacZ gene transfer. This would indicate that the MMP-targeted adenovirus has a decreased uptake into the liver. This was proven using the TaqMan quantitative RT-PCR TIMP-1 expression analysis. Animals with AdTIMP-1 gene transfer had a nearly four fold higher TIMP-1 expression level in the liver than the animals transduced with the modified AdTIMP-1(HWGF).

Efficacy of combination gene therapy (II)

In this study VEGF receptors 2 and 3 were stimulated and the activity of MMPs was inhibited using adenoviruses encoding VEGF-C and TIMP-1 respectively. We also over-stimulated the VEGF receptors 1, 2 and 3 using adenoviruses VEGF-A and VEGF-C encoding adenoviruses. A rabbit restenosis model was used for *in vivo* experiments. For two and four week time points we had six study groups; AdTIMP-1, AdVEGF-C, AdVEGF-A, AdlacZ, Ad(TIMP-1+VEGF-C) and Ad(VEGF-A+VEGF-C). At the two week time point the control AdlacZ group had the highest I/M ratio (0.56 ± 0.02), whereas AdTIMP-1 (0.37 ± 0.07), AdVEGF-C (0.39 ± 0.02), AdVEGF-A (0.44 ± 0.12) Ad(TIMP-1+VEGF-C) (0.38 ± 0.08) and Ad(VEGF-A+VEGF-C) (0.20 ± 0.07) showed reduced values for intimal thickening (figure 8). Statistical analysis showed a significant difference between the AdlacZ and the Ad(VEGF-A+VEGF-C) groups ($P<0.05$). At the four week time point AdlacZ control group and Ad(VEGF-A+VEGF-C) had the highest I/M ratio being (0.82 ± 0.18) and (0.86 ± 0.14), respectively. AdTIMP-1(0.39 ± 0.03), AdVEGF-C(0.47 ± 0.17), AdVEGF-A (0.73 ± 0.26), and Ad(TIMP-1+VEGF-C) (0.43 ± 0.01) groups showed decreased values for intimal thickening (figure 7). Significant differences in the I/M ratio were found between the control AdlacZ group and the AdTIMP-1 and the Ad(TIMP-1+VEGF-C) groups ($P<0.05$).

Balloon denudation caused endothelial damage in all rabbits. Endothelial coverage was measured in every group (being approximately 44% at the four week time point in every treatment group), but no statistically significant differences were found. RAM-11 stainings showed no inflammation or macrophage accumulation at the two week time point, whereas at the four week time point macrophage accumulation was clearly detectable in every group, being nearly two-fold higher when compared to the two week time point. This was especially clear in the Ad(TIMP-1+VEGF-C) group (data not shown). By specific RT-PCR, expression of all transgenes was detected in aortas two and four weeks after the gene transfer.

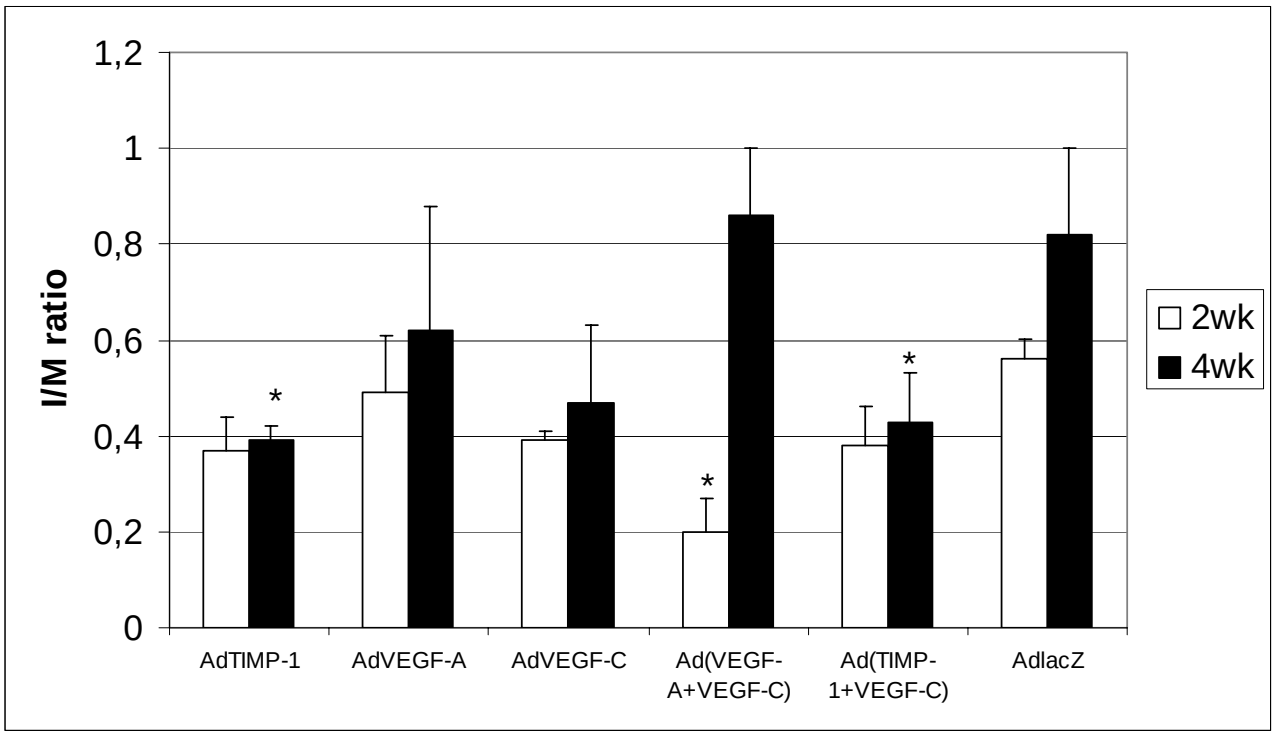

Figure 7. Intima/media ratios in combination studies at two and four week time points. *= P<0.05

Anti-oxidative gene transfer effect (III)

In this study the lipoprotein-associated phospholipase A_2 (PAF-AH) effects restenosis in rabbits was examined with intravascular adenovirus-mediated gene transfer. PAF-AH inactivates oxidized phospholipids and could therefore, potentially be an anti-inflammatory and antiatherogenic enzyme. A rabbit restenosis model was used for animal work. Histological analyses showed significantly ($p< 0.005$) reduced neo-intimal hyperplasia in the PAF-AH (0.25±0.03) group when compared to the lacZ controls (0.45±0.05) at the two week time point. At the four week time point, the difference between the PAF-AH group (0.34±0.05) and the lacZ group (0.53±0.06) was still detected, showing a tendency towards reduced restenosis ($p=0.057$). The RAM-11

staining showed no significant differences in macrophage accumulation between the groups. No significant differences were found between the groups in the regrowth of the endothelium. The percentage of proliferating cells was analyzed by BrdU labelling. No statistical differences were detected at the two week time point, but the PAF-AH group tended to have a lower proliferation rate than the lacZ group. At the four week time point the difference in proliferation between PAF-AH and lacZ was significant ($p<0.05$). The expression of PAF-AH was detected using RT-PCR.

Blood samples were collected at time points 0, 3, 7, 14, 21 and 28 days after the gene transfer. Plasma PAF-AH activity showed an increased statistical difference between the PAF-AH group and lacZ at the 14 day time point, but total cholesterol, triglycerides and ASAT did not show any significant changes. The LacZ group showed a higher CRP level at the three day time point, but it was not statistically significant. Also, the functionality of the PAF-AH was verified at mRNA, protein and enzyme activity levels in vitro and *in vivo*. Adenovirus distribution was determined by X-gal staining from the liver, spleen, kidney, lung and aorta. X-gal staining was seen in the spleen, liver and aorta two weeks after the gene transfer.

5.2. Gene therapy for vein graft stenosis in a rabbit model (IV)

Gene transfer studies in vein grafts were performed with Ad35K, AdTIMP-1 and AdLacZ at a viral titer of 1.0×10^9 pfu/m using a rabbit vein graft model. The expression of all transduced genes was detected in the vein grafts with RT-PCR. To determine the effects of gene transfers on neointima formation I/M ratios were measured. No differences were found in I/M ratios three days after the gene transfer. At the three day time point the I/M ratios were (0.19±0.04) in the Ad35K group and (0.24±0.03) in the AdlacZ group. Significantly ($p< 0.05$) reduced neointimal hyperplasia was found in the Ad35K group (0.24±0.04) at the two week time point when compared to the AdLacZ group (0.42±0.05), whereas the I/M in the AdTIMP-1 (0.30±0.1) group was not statistically significant . At the four week time point the I/M ratios were similar in all groups: AdLacZ (0.37±0.07), Ad35K (0.30±0.05) and AdTIMP-1 (0.32±0.04).

Macrophage accumulation is part of the inflammatory response in vein grafts. At the three day time point no macrophages were present, probably due to the short period of time after the surgery. At the two week time point there were no differences in the macrophage count. However, we noticed that macrophage accumulation was significantly lower in the Ad35K group at the four week time point when compared to the AdlacZ group. Endothelial coverage in the vein grafts after the gene transfer was analyzed by measuring the length of the intact endothelium from histological sections. No differences were found three days after the operation. At the

two week time point the endothelial coverage tended to be higher in the Ad35K group (95%±4.9) but the difference was not statistically significant. At the four week time point no statistically significant differences were present.

The percentage of proliferating cells was measured by BrdU labelling. At the three day time point no SMC proliferation was detected which could be due to the short time point after the operation. There were no statistically significant differences between the groups at the two week time point, but at the four week time point the AdTIMP-1 group (0.42%±0.11) showed a significant difference ($p<0.05$) when compared to AdlacZ (0.70%±0.3). On the other hand, the Ad35K and AdTIMP-1 groups showed significantly decreased apoptosis at the four week time point when compared to the AdlacZ group. At the three day time point no apoptosis was detected, due the short time period after the operation.

6. DISCUSSION

6.1. Gene therapy for restenosis with alternative treatment approaches

Restenosis is a major clinical problem after bypass operations and angioplasty since restenosis is detected in 30% to 60% of the patients within six months of the procedure (Serruys et al., 2002). De-endothelization after injury exposes subendothelial structures to circulating blood elements, leading to infiltrating monocyte, macrophage and lymphocyte accumulation in the vessel wall. In addition, arterial wall damage leads to platelet adhesion and aggregation and the generation of metal ions, iron and copper, that can facilitate LDL oxidation (Evans et al., 1995). Oxidizing species generated by damaged endothelium and activated platelets at the angioplasty site can induce chain reactions which result in endothelial dysfunction and LDL oxidation (Kugiyama et al., 1990; Steinberg et al., 1989). The activation of macrophages by oxidized LDL and the dysfunction of the endothelium can result in the release of growth factors promoting tissue proliferation. These changes, and also the release of the growth factors, cause SMCs to migrate to intimae.

It is recognized that endothelial damages and SMC migration and proliferation in the arterial wall are key elements in the pathogenesis of restenosis (Wilensky et al., 1995; Yutani et al., 1999). Also, angioplasty causes the formation of reactive oxygen species which impairs endothelial functioning causing oxidative stress. This would imply that anti-oxidative genes would also be beneficial in reducing restenosis (Laukkanen et al., 2002). Thus, restenosis is a complex process where interventions directed simultaneously to more than one gene could be more efficient than single treatments. Variety gene therapy approaches have already been studied for the treatment of restenosis (Rutanen et al., 2002a). However, complete consensus has not been reached. To have a better understanding of the pathogenesis of the restenosis we tested treatment genes and their combinations in a rabbit restenosis model (Hiltunen et al., 2000a) (studies I-III).

Adenoviruses were used in all of our studies as a gene transfer vector. Adenoviruses are the most widely used vectors due to their capacity to offer efficient transient gene expression to both proliferating and non-proliferating cells. Previous studies have shown that adenoviruses mediate an efficient transduction lasting two weeks (Hiltunen et al., 2000a). After that, the transduction efficacy diminishes and treatment groups rapidly catch up with the control group I/M levels. Also, systemic biodistribution is a common problem with adenoviruses. Intravascular transduction releases adenoviruses into systemic circulation and peripheral organs are transduced (Hiltunen et al., 2000b). Therefore, modifying the adenoviral vector would be beneficial in overcoming these barriers. In the first study, a selective gelatinase inhibitor (which was also the targeting peptide) was bound to the surface of the adenoviral vector.

Re-targeting adenoviruses alters tropism and enhances efficacy (I)

In my studies, TIMP-1 was used as a treatment gene and a cyclic MMP-2 and -9 targeting peptide (selective gelatinase inhibitor) was incorporated via a poly-lysine spacer to the adenovirus. Therefore, a dual effect in inhibiting the MMP function was used. It was noticed that the modified adenovirus had an altered tropism *in vitro* and *in vivo*. It is known that RAASMCs and EaHy cells have low levels of CAR receptors whereas the hepatocyte cell line Hep-G2 has high levels of CAR-receptors (Biermann et al., 2001; Fechner et al., 1999). In *in vitro* studies it was found that the peptide-modification increased adenovirus transduction efficiency in RAASMC and EaHy cells but decreased transduction efficiency in the Hep-G2 cell line. The different behaviour of the viral complexes in cells originating from the liver and vascular wall could be due to the different entry mechanisms of the vectors into these cells and the levels of the CAR receptor in that cell line. Hidaka *et al.* have shown that fibre-altered Ad5-based vectors demonstrated enhanced gene transfer efficiency in CAR-deficient fibroblasts, with no further enhancement in CAR-sufficient fibroblasts (Hidaka et al., 1999). They demonstrated that CAR deficiency in the target cells can be circumvented either by supplying CAR or by modifying the adenovirus fibre to bind to other cell-surface receptors.

Printz *et al.* have also shown that the fibroblast growth factor receptor retargeted adenovirus also had an altered biodistribution after i.v. injection and that the liver showed markedly decreased transduction efficiency (Printz et al., 2000). In my study, the transglutaminase treatment likely changed the adenovirus surface in such a manner that the natural entry via the CAR receptor is diminished. Our results are in line with previously published data that the transglutaminase reaction itself increases the transduction efficiency of adenoviruses (Wagner et al., 1992).

Even though MMPs are secreted enzymes, these proteins may form complexes on the cell surface with $\alpha_v\beta_3$ intergrins (Brooks et al., 1996). These complexes are stable enough to mediate targeting of the HWGF adenoviruses. Non-modified adenoviruses typically used in gene therapy trials have a very wide tropism primarily due to the expression of CAR and $\alpha_v\beta_3$ and $\alpha_v\beta_5$-integrins (Ylä-Herttuala and Martin, 2000). This is a clear drawback since target tissue-restricted gene expression would be a safety advantage in human gene therapy. Physical targeting of gene transfer, especially in the arterial system, for example with catheters, is feasible but still leads to biodistribution of the transgene to peripheral organs (Hiltunen et al., 2000b). Thus, reducing the inadvertent adenovirus uptake to the liver via peptide modification of the virus surface should improve the safety profile of the adenoviruses. Tissue or disease specific promoters could further improve the outcome (Ylä-Herttuala and Martin, 2000). Thus, modification of the adenoviruses with specific targeting

peptides provides a promising alternative to improve the specificity and safety of adenovirus mediated gene transfer *in vivo*.

Does combination therapy improve results? (II)

In this study two different pathological steps of restenosis were simultaneously affected. SMC migration was inhibited using TIMP-1 gene transfer. Simultaneously, re-endothelialization was stimulated via VEGFR-1 and VEGFR-2 and VEGFR-3 using VEGF-A and VEGF-C as treatment genes. It was found that th synergistic combination of VEGF-A and VEGF-C was the most effective at the two week time point. This could probably be explained by the rapid over-expression of the endothelium stimulating factors. However, balloon denudation also initiated inflammatory and prolifetarory processes in the vessel which the VEGFs did not inhibit, which would explain the high I/M levels at the four week time point in the VEGF combination group and also the macrophage increase at the four week time point. Actually, it is believed that VEGF induces migration and activation of monocytes (Barleon et al., 1996) and VEGF-A has been shown to upregulate MMP production (Wang and Keiser, 1998) which itself causes SMC proliferation and migration. Also, *Zhao et al* have demonstrated that blocking VEGF in vivo in mice inhibited early inflammation and later neointimal formation, suggesting that VEGF might promote neointimal formation by acting as a proinflammatory cytokine (Zhao et al., 2004a). Moreover, Zhao et al suggested that increased VEGF and its receptors would act directly on smooth muscle cells, resulting in structural changes such as medial thickening (Zhao et al., 2004b). Also, a few studies have reported that VEGF has direct actions on the proliferation/migration of smooth muscle cells which may not be mediated by inflammation (monocyte recruitment) (Wang and Keiser, 1998; Ishida et al., 2001). Therefore the use of VEGF-A would not be the optimal choice for restenosis combination studies.

Recently, Hutter et al showed that the production of endogenous VEGF is crucial in terms of inhibiting neointima formation. They blocked endogenous VEGF production using a VEGF-trap. Sequestration of endogenous VEGF by the VEGF-trap delayed reendothelialization and dramatically increased neointimal size, implicating the VEGF pathway in the control of the arterial repair process (Hutter et al., 2004). They suggested that delayed reendothelialization may initiate a complex cellular and molecular cascade that eventually results in a change of neointima size. Also, Khurana et al recently showed that neointimal formation after mechanical injury of an artery consists both of angiogenesis-dependent and independent components. The mechanical injury to the artery by itself induces a mild adventitial angiogenic response. The augmentation of this response led to a greatly increased neointima formation, whereas even full inhibition of adventitial neovascularization did not eliminate the injury-induced component of neointima development (Khurana et al., 2004b).

When considering the use of VEGFs in the treatment of restenosis one has to bear in mind that controversial data has been published on VEGF effects on restenosis and neointima formation. The positive effect of VEGF in decreasing neointima formation has been noticed (Hiltunen et al., 2000a; Rutanen et al., 2002b; Khurana et al., 2004a). There has, however, also been a few negative studies considering the inflammatory effects mediated by VEGFs (Ohtani et al., 2004). The big vector load, as used here, may promote the inflammation and the neointima formation in the long run whereas the beneficial effects have been noticed when small doses are being used (Bhardwaj et al., 2003). Also, liposome-mediated gene transfer has been shown to reduce neointimal formation in collar model of rabbits and also inhibiting macrophage accumulation partly through an indirect neointima-decreasing effect (Khurana et al., 2004a). Kuharana et al discussed whether the proatherosclerotic effects of VEGF may require at least transiently high systemic levels of VEGF and be mediated indirectly via the nonendothelial actions of VEGF. In this study the vector load is nearly 30 times higher when compared to the former study. The difference may be enough to mediate the adenoviral inflammation process and the non-beneficial behaviours of the VEGF gene in the vessel wall. Actually, in the intramyocardial gene transfer model, Rutanen and colleagues have showed that high VEGF doses cause myocardial oedema and pericardial fluid accumulation (Rutanen et al., 2004). They suggested that the correct dose of VEGF is a very important factor in achieving acquired results.

This is the first study to compare single and combination therapies for the treatment of restenosis. When considering genes tested in this study, the TIMP-1 gene transfer is alone sufficient for decreasing neointima formation. The data is in line with previous publications with TIMP-1 restenosis studies (Dollery et al., 1999; Forough et al., 1996). These findings suggest that VEGFs may not be the ideal combination for restenosis gene therapy.

The anti-oxidant gene therapy approach (III)

Arterial damage after angioplasty results in the formation of PAF, which activates platelets and promotes neutrophil adhesion to endothelial cells (Eldar et al., 1992). PAF also induces the production of a range of inflammatory cytokines and is a mitogen for vascular SMCs (Evangelou, 1994). The pathophysiological effects of PAF in vivo are mainly regulated by PAF-AH, which inactivates PAF. In addition to PAF, PAF-AH can effectively hydrolyze biologically potent oxidized phospholipids resembling the structure of PAF (Stremler et al., 1991).

In this study it was shown that adenovirus mediated PAF-AH gene transfer results in a significant inhibition of neointima formation in balloon denuded rabbit aortas and the therapeutic effect was still detected at the four week time point. The first evidence of an inhibitory effect of PAF-AH on intimal hyperplasia was shown by Quarck et al, who demonstrated that the adenovirus-mediated gene transfer of human PAF-AH inhibited injury-induced neointima formation and resulted in reduced adhesion molecule expression and monocyte adhesion in apolipoprotein E-deficient mice (Quarck et al., 2001). The protective effect of PAF-AH on restenosis may be due to its ability to remove PAF-like oxidized phospholipids formed during LDL oxidation. There are several reports demonstrating the generation of PAF-like lipids during the oxidation of LDL which can activate PAF receptors (Tokumura et al., 1996; Heery et al., 1995). Oxidized lipoproteins inhibit the release of NO from endothelial cells (Chin et al., 1992). In addition, oxidative stress exerts toxic effects on vascular SMCs, leading to the activation of monocytes and macrophages, which initiate a cascade of inflammatory responses (Berliner et al., 1995). Reactive oxygen species and lipid oxidative products induced by balloon injury have been reported to alter growth factor production, which could also influence SMC proliferation (Ku et al., 1988). In this study the proliferation index was significantly decreased in the PAF-AH group at the four week time point which could be due the anti-apoptotic effects of PAF-AH (Matsuzawa et al., 1997). It is believed that apoptotic cells release cytokines which increase the proliferative response (Walsh et al., 2000).

A couple of studies have suggested that PAF-AH levels in blood may indicate an elevated risk for CHD (Satoh et al., 1992; Packard et al., 2000). However, the high PAF-AH levels in the blood are just a reaction to high blood LDL-levels due to the capability of PAF-AH to neutralize the oxLDL (Kovanen and Pentikainen, 2003). Therefore, the high levels of PAF-AH in the blood would indicate increased risk for CHD. The beneficial results from this study may be explained by local gene delivery. The oxLDL enhances the LDL retention by the endothelial cell matrix bound lipoprotein lipase (Auerbach et al., 1996). Using anti-oxidant PAF-AH as a treatment gene the local downregulation of oxLDL in the vessel wall is achieved. This may lead to the local inhibition of cytokine release and therefore also the lowered proliferation rate of SMCs. Furthermore, there are a few trials in which antioxidants have been used for preventing restenosis (DeMaio et al., 1992; Yokoi et al., 1997; Tardif et al., 2003). These trials indicated the use of antioxidants after PTCA. However, further studies are needed to examine the long term effects of PAF-AH gene transfer in restenosis. It is concluded that local intravascular delivery of PAF-AH decreases neointima formation in a rabbit model.

6.2. Gene therapy for vein graft stenosis in a rabbit model

Vein grafts are widely used for coronary artery bypass surgery. The operation is highly effective in relieving the symptoms of angina and prolonging life quality. Unfortunately, the occlusion of venous bypass grafts still remains a significant problem. The pathogenesis of vein graft stenosis is quite similar to that of restenosis where the formation of neointima is concerned. Intimal hyperplasia is caused by SMC proliferation and migration. The process is induced by cytokines and growth factors. In contrast to the arterial injury models, in venous grafts the major portion of the intimal hyperplasia occurs after the endothelial regeneration. Vein graft stenosis is also a complicated process, leading to graft occlusion in 50% of the patients ten years after the operation (Fitzgibbon et al., 1996). An *Ex vivo* method was used for the gene transfer and therefore no systemic distribution of the vector was exposed to the circulation.

Although a number of studies have addressed molecular and cellular events in arterial restenosis, very little is known about gene transfer to vein grafts. Mann et al showed that decoy oligonucleotides against E2F transcription factor reduced stenosis in human vein grafts (Mann et al., 1999). However, the phase three multicenter PREVENT III results (vein graft used for peripheral artery bypass) were negative The E2F decoy failed to show it's efficacy over the 12 months following the surgery (http://www.medicalnewstoday.com/medicalnews.php?newsid=17439). Also, the E2F treatment has shown its efficacy in coronary vein grafts (Grube, 2001). The results of the phase III multicenter trial for CABG patients (PREVENT IV) have not yet been published. Accordingly, Ehsan et al have shown that there is a long term stabilization of vein graft wall architecture and prolonged resistance to experimental atherosclerosis after E2F decoy oliconucleotide gene therapy in a rabbit model (Ehsan et al., 2001). Chen et al used the adenoviral gene transfer of soluble VCAM in porcine interposition vein grafts, which reduced neointimal thickening (Chen et al., 1994). It has also been shown that sdi-1, a mediator of tumour suppressor p53 action, inhibited neointima formation in rabbit vein grafts (Bai et al., 1998). George et al have studied the effects of TIMPs on human saphenous veins. TIMP-2 inhibited neointima formation in organ cultures ex vivo but no significant effect was noticed in porcine jugular vein grafts *in vivo* (George et al., 1998a). However, TIMP-3 was found to inhibit neointima formation in a porcine vein graft model (George et al., 2000). Also, the adenoviral gene transfer of eNOS has been reported to reduce neointima formation (Cable et al., 1997). To the best of my knowledge, this was the first study to compare different genes in the rabbit jugular vein graft model.

Anti-inflammatory protein 35K is effective in decreasing vein graft stenosis (IV)

The hypothesis was that gene transfers with Ad35K and AdTIMP-1 during a vein graft operation might be effective in reducing neointimal thickening. Since gene transfer was made *ex vivo* no vector was likely to have been exposed to systemic circulation. The selection of therapeutic genes was based on the pathophysiological mechanisms of stenosis. Vaccinia virus anti-inflammatory protein 35K was selected because it is known to bind CC-chemokines like MCP-1 efficiently (Bursill et al., 2004). It was assumed that 35K would inhibit inflammatory responses and macrophage accumulation. Intimal hyperplasia can be considered a vascular wound healing response after vein grafting where monocytes and macrophages have a central role (Eslami et al., 2001). It has been shown that the MCP-1 gene is upregulated soon after surgery, suggesting a central role for CC-chemokines in intimal hyperplasia (Stark et al., 1997). The AdTIMP-1 group also served as a positive control and helped to measure the magnitude of the effect obtained by Ad35K which had not been previously used for the treatment of restenosis or vein graft thickening.

It was found that the anti-inflammatory protein 35K significantly reduced neointima formation at the two week time point when compared to the control. TIMP-1 also showed a tendency towards reduced neointima formation but was not statistically significant. However, at the four week time point all the treatment groups had reached the control group values in the I/M ratio. Thus, only short term effects were achieved, which may be due to the transient nature of the adenoviral gene transfer effect (Hiltunen et al., 2000a).

It was also found that macrophage accumulation was significantly decreased at the four week time point in the Ad35K group when compared to the AdlacZ group. Also, the Ad35K group showed a tendency towards a decreased SMC proliferation index at a four week time point. This may be due to the anti-inflammatory properties of 35K which delays the overall inflammation response in the vessel wall. Therefore, it's not unexpected that changes in the proliferation index and the macrophage accumulation can be seen at a four week time point. 35K can block the CC cytokines which reduce macrophage accumulation and the SMC proliferation rate (Bursill et al., 2003). However, the AdTIMP-1 group had a significantly decreased proliferation rate at the four week time point which could be due to the over-expression of TIMP-1. It looks like that the neointima formation needs at least two cascades: the inflammatory and the proliferation cascade. Using 35K gene transfer the inflammatory and to some extent, the proliferation cascade are inhibited whereas with TIMP-1 gene transfers only the proliferation cascade is inhibited.

Since macrophages express several MMPs (Libby et al., 1992; Zempo et al., 1994; Bendeck et al., 1994), the balance between MMPs and TIMP in the vein graft may be favourably altered. After two weeks the adenoviral

expression rate decreases and the inhibitory effect of the over-expressed TIMP-1 disappears, potentially explaining the decreased proliferation index at the four week time point. At the three day time point, signs of SMC proliferation or macrophage accumulation were not yet detectable, which is probably due to the short time period after the operation. Decreased apoptosis was evident at the four week time point both in the Ad35K and AdTIMP-1 groups compared to the control group. It has been previously shown that early inhibition of apoptosis reduces neointima formation after balloon injuries. Therefore, the decreased apoptosis rate could reflect the additive effects of decreased inflammation and lower rate of SMC proliferation and macrophage accumulation (Beohar et al., 2004).

In terms of decreasing vein graft stenosis, it might be that the earlier the pathogenesis of vein graft stenosis is inhibited the more effective the results be. In other words, the inflammation process that emerges needs to be inhibited or delayed and in that way the proliferation processes are also delayed. Laukkanen et al have shown the efficacy of the anti-inflammatory protein Ec-SOD in a restenosis model (Laukkanen et al., 2002). Therefore, it is likely that 35K would also mediate similar effects. Furthermore, combination studies (for example with the TIMP-1 gene) would be one future research choice both for restenosis and vein graft studies. It is concluded that the anti-inflammatory effects of 35K may be useful for the inhibition of vein graft stenosis. Also, its ability to decrease macrophage accumulation and SMC proliferation could be beneficial in the treatment of vein graft stenosis.

6.3. Which method to use?

These findings suggest that TIMP-1 gene transfer can have beneficial effects both in restenosis and vein graft stenosis models. When the advantages of re-targeting adenoviruses to non-modified adenoviruses are compared, the systemic biodistribution is clearly a drawback with non-modified adenoviruses. A four-time lower transduction level was detected in the livers of rabbits treated with modified adenoviruses. Also, it was found that targeting the TIMP-1 encoding adenovirus with the MMP-2 and MMP-9 binding peptide motif (HWGF) decreased neointima formation at least as efficiently as intact AdTIMP-1.

In the combination study, the over-expression of TIMP-1 inhibited the migration of SMCs and combined with VEGF-C, a longer treatment effect was achieved. However, TIMP-1 gene transfer alone showed prolonged treatment efficacy suggesting that inhibiting SMC migration might be more crucial to the early pathogenesis of restenosis than than affecting endothelial functions with VEGFs. Also, it is possible that the endogenous VEGF

production is enough in terms of reducing neointima formation, given that VEGFs promote cell proliferation and stimulate pro-inflammatory components, and that therefore, combination gene therapies using high titers of VEGFs are not needed for the treatment of restenosis.

Controversial data regarding TIMP-1's role in inhibiting SMC proliferation has been published. Forough et al found that TIMP-1 inhibits SMC proliferation (Forough et al., 1996). Akahane published contradictory data suggesting TIMP-1 has a role in promoting proliferation (Akahane et al., 2004). Also, results on TIMP-1 with no impact on SMC proliferation have been published (George et al., 1998b; Baker et al., 1998). The vein graft studies showed that TIMP-1 adenoviral gene transfer reduced SMC proliferation. Therefore, it may be that TIMP-1 gene transfer has an indirect net effect inhibiting MMP production which results in a lowered proliferation rate of SMCs and decreased neointima formation.

On the other hand, the first study (study I) showed promising results with regard to targeting peptides. It might be that MMP-targeted TIMP-1 gene transfer would be the best option for decreasing restenosis and vein graft stenosis when safety issues are also considered. However, long-term treatment results might be achieved if TIMP-1 is combined with anti-inflammatory protein gene transfer. Anti-inflammatory proteins have shown great promise decreasing neointima formation even at longer time points (Laukkanen et al., 2002). Also PAF-AH gene transfer showed a tendency towards long term treatment results (study III). This way we would both inhibit the early inflammation and the proliferation processes. Targeting the vectors and using combination therapy could also be an interesting approach.

PAF-AH gene transfer gave promising results decreasing restenosis. Similar results should be expected with 35K gene transfer in neointima formation. Because of the injury model of restenosis, the inflammation process is more drastic than in vein graft stenosis and therefore, further studies with 35K would be appropriate as far as restenosis studies are concerned. However, it is not yet known which way 35K mediates its effects. Bursill et al suggested three different ways. Firstly, 35K would bind circulating CC-CKs. Secondly, it may bind CC-CKs on the endothelial cell surface and strip the CC-CKs from the endothelia. Or thirdly, 35K would remain bound to the CC-CKs preventing interaction with circulating monocytes (Bursill et al., 2004). In our restenosis rabbit model the endothelia is injured and therefore the binding area of chemochines would be decreased, having either a positive or negative affect on the results. Also, the efficacy of 35K gene transfer may be improved via vector targeting or with the use of another vector. For example AAVs mediate stable transduction for as long as several months, which could be a beneficial property as far as further studies are concerned.

Taken together, these studies show that various means can be used to prevent vascular thickening in animal models. In my opinion, combination gene therapy holds great promise for the future prevention of restenosis and vein graft stenosis. However, the optimal gene cocktail still needs to be discovered. Due to the differences between animal models and the human pathogenesis of these diseases, further studies are needed to evaluate the efficacy of these treatment methods in clinical use.

7. FUTURE PROSPECTS

There remains a growing interest in gene therapy with regards to cancer and vascular disease treatment. Considering the present treatment methods available, it is difficult to get effective and long lasting results. Effective gene transfer vectors are needed for efficient gene transfer and long-term expression, which is especially necessary for cancer therapy. Re-targeting viral vectors, for example, to the specific cells using targeting moieties has been one development in the field. Also, developing new viral vectors with the multiple qualities of promoters and core modifications for targeting, will allow us to achieve more efficient and more disease specific results. In that way the specific tissue, organ or cells would get the maximum treatment without systemic spread and toxic reactions.

Combination gene therapy is a potential future method for getting the most effective therapeutic results. Using gene cocktails to overcome two or three steps in the pathogenesis of vascular diseases, long lasting treatment results could be achieved. However, large viral loads with systemic spread would still be a problem. These problems may be overcome by re-targeting vectors or using local gene transfer where the vector is not allowed to spread to systemic circulation.

Also, the pathogenesis of these diseases requires further study. As far as these studies are considered, it appears that the earlier the pathogenesis of these diseases is discovered and the earlier intervention takes place, the better the results. Another problem is how to get the same positive results in clinical studies as in preclinical studies with animals? It is necessary to make animal models as humanlike as possible, perhaps by using genetic models e.g. transgenic animals, before taking treatment into the clinic. On the other hand, efficient post-operational dietary restrictions and optimal medication would be the first and primary choice for the prevention, progression and formation of atherosclerotic lesions. In the meantime continuous research is underway to treat these diseases which are mostly the result of lifestyle choices.

8. SUMMARY AND CONCLUSIONS

Atherosclerosis is the most common disease in developed countries. In Finland it is the biggest cause of mortality and morbidity. Current treatment methods include lifestyle modifications and medication and in the most severe cases angioplasty or bypass operations are considered. The aim of this study was to investigate gene therapy for restenosis and vein graft stenosis.

A modified adenovirus was developed linking an MMP-2 and MMP-9 targeting peptide on the surface of the adenovirus. The aim was to find out whether targeting increases the transduction efficacy of adenoviruses. It was found that HWGF peptide modification increased the transduction efficacy of the adenovirus mediated gene transfer in SMCs and ECs in *in vitro* and enhanced gene transfer to the arterial wall *in vivo*. Also, peptide modification of adenoviruses beneficially modulated tissue tropism *in vivo*. TIMP-1 gene transfer reduced intimal thickening in an established restenosis model in rabbits. Further, the effect of the lipoprotein-associated phospholipase A_2 (PAF-AH) on restenosis was studied in rabbits with intravascular adenovirus-mediated gene transfer. It was found that local catheter-mediated delivery of PAF-AH adenoviruses can reduce restenosis in rabbits.

A rabbit restenosis model was used to find out whether combination gene therapy is more effective than single gene therapy. The VEGF-A and VEGF-C combination was the most effective at the two week time point. However, TIMP-1 gene transfer alone and simultaneous utilization of two different pathogenetic mechanisms via VEGF receptors and MMPs using TIMP-1 and VEGF-C encoding adenoviruses prolonged the treatment effect. It is concluded that TIMP-1 gene transfer is sufficient for the treatment of restenosis on its own.

Treatment of vein graft stenosis was studied in a rabbit model. TIMP-1 and 35K were used as treatment genes. It was demonstrated that the anti-inflammatory effects of 35K may be useful for the inhibition of vein graft stenosis. Also, its ability to decrease macrophage accumulation and SMC proliferation could be beneficial in the treatment of vein graft stenosis.

In conclusion, various treatment methods was used to prevent vascular thickening. Retargeted adenoviruses showed reduced uptake into the liver. Further, anti-inflammatory protein and anti-oxidant protein effectively reduced neointima formation. Also, combination gene therapy gave encouraging results. However, the optimal gene cocktail still needs to be evaluated. These findings suggest that gene therapy can be used for a therapeutic approach to vascular thickening.

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