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PÄIVI TURUNEN

Gene Therapy of Restenosis and Vein Graft Disease

Studies with Single Genes and Gene Combinations

Doctoral dissertation

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ABSTRACT

Cardiovascular disease is the leading cause of illness and death in developed countries. The majority of cases stem from atherosclerosis, which involves multiple processes including endothelial dysfunction, inflammation, vascular proliferation and matrix alteration. Treatment of cardiovascular diseases consists of a wide variety of interventions from life style changes to surgery. Conventional therapies for the treatment of cardiovascular disease have focused on the use of angioplasty (PTCA), stenting and heart bypass surgery, and the reduction of blood pressure and cholesterol through the use of medication, along with lifestyle changes in exercise, diet and stress management. In this study many potential treatment genes were tested in vitro and in vivo animal models to find new strategies to prevent restenosis after PTCA and vein grafting.

Oxidized low density lipoprotein (oxLDL) plays an important role in atherosclerosis. Oxidative modification of LDL generates biologically active platelet activating factor (PAF)-like phospholipid derivatives with polar fatty acid chains, which have potent proinflammatory activity. These products are inactivated by lipoprotein-associated phospholipase A_2 (Lp-PLA_2), an enzyme capable of hydrolysing PAF and PAF-like phospholipids. In this study it was shown that adenovirus-mediated Lp-PLA_2 gene transfer leads to overexpression of Lp-PLA_2 in liver and in vivo production of LDL particles with increased Lp-PLA_2 activity and protected LDL particles from degradation and decreased foam cell formation in vitro. It was also showed that adenovirus-mediated Lp-PLA_2 gene transfer can reduce restenosis after balloon denudation in rabbits.

Vein graft stenosis is a common problem after bypass surgery. Vein grafts are ideal targets for gene therapy because transduction can be made ex vivo before grafting. Since chemokines and inflammatory factors are involved in vein graft thickening we tested a hypothesis that vaccinia virus anti-inflammatory protein 35k, which can sequester almost all CC-chemokines, can reduce vein graft thickening in vivo. It was found that the anti-inflammatory protein 35k was an efficient factor in reducing neointima formation, macrophage accumulation and proliferation in rabbit vein grafts after adenoviral ex vivo gene transfer. In next study we used the combination gene therapy in order to reduce neointima formation more efficiently and prolong the treatment effect in vein grafts. We also wanted to study the effects of anti-inflammatory EC-SOD, which has shown a great promise decreasing restenosis in a rabbit model. It was shown that the combination had favourable effects. The combination of EC-SOD+35k and EC-SOD+TIMP-1 resulted in more enhanced inhibition of neointima formation than TIMP-1 or 35k alone. When combining two anti-inflammatory genes EC-SOD+35k it led to significant decrease in macrophage count comparing to single gene transfer results. Also, AdEC-SOD alone showed significant anti-inflammatory effects suggesting its persistent anti-inflammatory effect. These findings indicate that oxidative stress may play an important role in the pathogenesis of vein graft stenosis.

It is concluded that atherogenic properties of oxLDL is reduced when increasing content of Lp-PLA_2. Adenovirus-mediated gene transfer of Lp-PLA_2 also reduces restenosis after balloon denudation. In a rabbit model of vein graft disease combination gene therapy of anti-inflammatory and anti-proliferative proteins was effective in decreasing neointima formation. This may be because two different treatment genes can more efficiently affect to the pathological events at early stages and stop the degenerative domino effect more efficiently than just one gene transfer.

National Library of Medicine Classification: QU136, QY58, QZ180, WG169, WG300
Medical Subject Headings: angioplasty, transluminal/adverse effects; coronary artery bypass/adverse effects; coronary restenosis/prevention and control; gene therapy; disease models; oxidative stress; oxidized low density lipoprotein; phospholipases A/metabolism

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ABBREVIATIONS

AAV	adeno associated virus
Ad	adenovirus
ASAT	aspartyl aminotransferase
AS-ODN	antisense-oligonucleotides
ATII	angiotensin II
BrdU	5'-bromo-2'-deoxyuridine
CABG	coronary artery bypass grafting
CE	cholesterol ester
CHD	coronary heart disease
CMV	cytomegalovirus
CRP	C-reactive protein
EC	endothelial cell
ECM	extracellular matrix
EC-SOD	extracellular superoxide dismutase
FGF	fibroblast growth factor
HDL	high density lipoprotein
HIV	human immunodeficiency virus
ICAM	intercellular adhesion molecule
IFN-γ	interferon-γ
IGF	insulin like growth factor
IL-1	interleukin-1
LDL	low density lipoprotein
Lp(a)	lipoprotein-a
Lp-PLA_2	lipoprotein-associated phospholipase A_2
MCP-1	monocyte chemoattractant protein-1
M-CSF	macrophage colony stimulating factor
MDA	malondialdehyde
MMP	matrix metalloproteinase
MOI	multiplicity of infection
NLS	nuclear localization signal
NO	nitric oxide
NZW	New Zealand white
PAF	platelet activating factor
PAF-AH	platelet activating factor-acetylhydrolase
PC	phosphatidylcholine
PDGF	platelet derived growth factor
PTCA	percutanueous transluminal coronary angioplasty
Rb	retinoblastoma
SMC	smooth muscle cell
SR	scavenger receptor
TF	tissue factor
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumor necrosis factor-α
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell
vWF	von Willebrand factor

LIST OF ORIGINAL PUBLICATIONS

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

I Turunen P, Jalkanen J, Heikura T, Puhakka HL, Karppi J, Nyyssönen K, Ylä-Herttuala S: Adenovirus-mediated gene transfer of Lp-PLA_2 reduces LDL degradation and foam-cell formation in vitro. J Lipid Res. Sep;45(9):1633-9, 2004

II Turunen P, Puhakka H, Rutanen J, Hiltunen MO, Heikura T, Gruchala M, Ylä-Herttuala S: Intravascular Adenovirus-Mediated Lipoprotein-Associated Phospholipase A_2 Gene Transfer Reduces Neointima Formation in Balloon-Denuded Rabbit Aorta. Atherosclerosis Mar;179(1):27-33, 2005. Epub 2004 Dec 13.

III Puhakka HL, Turunen P, Gruchala M, Bursill C, Heikura T, Vajanto I, Greaves DR, Channon K, Ylä-Herttuala S: Effects of Vaccinia Virus Anti-Inflammatory Protein 35k and TIMP-1 Gene Transfers on Vein Graft Stenosis in Rabbits. In Vivo 19(3), 2005 (in press)

IV Turunen P*, Puhakka H*, Heikura T, Romppanen E, Inkala M, Leppänen O, Ylä-Herttuala S: Combination Gene Therapy of EC-SOD with 35k or TIMP-1 in the Treatment of Vein Graft Stenosis in Rabbits.
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1. Introduction

Cardiovascular disease is the leading cause of illness and death in developed countries. Many risk factors can help to predict the likelihood of the disease: heredity, being male, age, cigarette smoking, high blood pressure, diabetes, obesity, lack of physical activity, and abnormal blood cholesterol level. The majority of cases stem from atherosclerosis, a condition in which cholesterol and fibrous tissue accumulate in the walls of large and medium-sized arteries. It involves multiple processes including endothelial dysfunction, inflammation, vascular proliferation and matrix alteration.

Treatment of cardiovascular diseases consists of a wide variety of interventions from life style changes to surgery. Conventional therapies for the treatment of cardiovascular disease have focused on the use of angioplasty (PTCA), stenting and heart bypass surgery, and the reduction of blood pressure and cholesterol through the use of medication, along with lifestyle changes in exercise, diet and stress management. However, during arterial manipulations the injury in the vessel induces synthesis of factors that stimulate smooth muscle cell (SMC) migration and proliferation and thrombogenic factors leading to intimal hyperplasia and restenosis, which can lead to re-occlusion of the treated vessel. Despite advances in pharmacological therapy new methods are needed to improve the prevention of progression of atherosclerosis and restenosis.

Gene therapy in the field of cardiovascular disease will be useful for the treatment of many pathological diseases, such as restenosis after angioplasty and vascular bypass graft occlusion. Comprehensive understanding of vascular biology and gene expression is important in selecting potentially therapeutic genes to express in the vascular wall. Experimental studies have established the proof of concept that gene transfer to cardiovascular system can achieve therapeutic effects, but there are some important issues to be solved, such as effective delivery tools.

In this study, potential treatment genes were tested in vitro and in vivo animal models to find new strategies to prevent restenosis after PTCA and vein grafting.

2. Review of the literature

2.1. Atherosclerosis

Atherosclerosis, with its clinical complications, for example stroke and myocardial infarction, is one of the main causes of morbidity and mortality in Western countries. Rupture of atherosclerotic plaques and subsequent occluding thrombus formation is the main cause of myocardial and cerebral infarction. Despite many advances in cardiology, atherosclerosis remains a major medical problem, especially in the case for individuals with insulin resistance and type 2 diabetes mellitus. Treatment of symptomatic coronary atherosclerotic plaques by angioplasty leads to vascular responses including neointimal formation and constrictive remodelling causing restenosis (Bittl, 1996).

2.2.1. Pathogenesis

Different cellular entities are involved in the pathobiology of atherogenesis. These cells can be organized into structural elements of the arterial wall (endothelial cells and vascular smooth muscle cells), inflammatory cells that enter the arterial wall, and circulating elements (e.g. platelets and leukocytes) (Glass and Witztum, 2001). Endothelial cells (ECs) transduce responses to pathogenic stimuli such as hypertension, hyperglycemia and cigarette smoke. One such critical endothelial response is the induction of adhesion molecules, a decisive step early in atherogenesis (Libby, 2000). Another important response is the change in the production of nitric oxide, that helps maintain the normal endothelial reactivity while limiting thrombosis and inflammation (Tedgui and Mallat, 2001). Medial SMCs in the arterial wall provide the essential structural component and help to maintain vascular tone. The migration of SMCs from the media to the intima and their subsequent proliferation is an important step in atherogenesis (Ross, 1993). Also, SMCs provide the main source for the extracellular matrix, which together with SMCs form the fibrous cap overlying the lipid core of atherosclerotic plagues. In addition, the SCMs stimulate matrix metalloproteinases (MMPs), which contribute to the remodelling process of the arterial wall (Libby, 2000). Atherosclerosis progresses in a series of stages, although some lesions at each stage may not progress further if inciting events, such as hypercholesterolemia, diabetes, smoking or hypertension, are controlled (Glass and Witztum, 2001).

The initial stage of atherogenesis involves the accumulation of lipoproteins in the subendothelial space in focal areas of the arterial tree, usually at branch points with disturbed laminar flow. In response to this retention, a series of biological responses

ensue, including lipoprotein modification, endothelial alterations, inflammatory responses including T-cell recruitment, cytokine secretion, monocyte chemotaxis and subendothelial macrophage accumulation, and intracellular cholesterol accumulation in macrophages. Much of the cholesterol is stored as cholesterol esters (CE) in cytoplasmic lipid droplets, which give the macrophages a foamy appearance when viewed by microscopy, and thus these cells are referred to as foam cells. The presence of macrophage foam cells defines the earliest pathological lesion, fatty streak (Ross, 1999).

Although sensitive tests of endothelial function show abnormalities in vasodilation in the very earliest phases of atherosclerosis (Vogel et al., 1998), fatty streaks are not occlusive and cause no clinical symptoms. However, some fatty streaks may progress over years to more complex lesions that can give rise to chronic symptoms and acute events. An important event in the progression of fatty streaks involves the migration of SMCs from the media to the intima and the secretion of large amounts of collagen and matrix proteins by these cells (Libby, 2000). In addition, macrophages proliferate and continue to accumulate more lipids. SMCs can also accumulate lipids and become foam cells. These events give rise to so called fibrous lesions, which are lesions consisting of lipid loaded macrophages and SMCs covered by a fibrous cap. Further progression to complex lesions involves the accumulation of extracellular lipids, which results from the combination of aggregation and fusion of matrix-retained lipoproteins and release of lipid droplets from dying foam cells. Calcification, hemorrhage, and microthrombi can also be observed in these complex lesions (Stary, 2000). If arterial occlusion increases gradually, the patient will experience exercise-induced ischemia. If the lesions rupture or erode before they become large and occlusive, acute vascular events, such as unstable angina, heart attacks, sudden death or strokes can occur. Rupture involves the abrupt disruption of the fibrous cap, followed by exposure of thrombogenic material and acute thrombosis. Importantly, rupture mostly occurs in lipid-rich and macrophage rich shoulder regions of the plaque with a thin fibrous cap and is probably triggered by the degradation of the fibrous cap by proteases secreted by macrophages or released from dying foam cells (Stary, 2000).

2.2.2. Low-density lipoprotein oxidation

The primary event in atherogenesis is apo-B containing lipoprotein deposition in the arterial wall. Low density lipoprotein (LDL) is the major cholesterol-carrying lipoprotein in plasma and is the causal agent in many forms of coronary heart disease (Salonen et al., 1991). Plasma lipoproteins continuously enter the subendothelial space of vessels via leakage through transient gaps between

endothelial cells and also via endothelial transcytosis. Under normal conditions, lipoproteins are not retained in the subendothelium and simply re-enter the circulation. In certain focal areas of the arterial tree, however, lipoprotein retention is increased, leading to their accumulation in the arterial wall (Williams and Tabas, 1995). In the intima LDL is exposed to oxidation. Agents capable of initiating lipid peroxidation include lipoxygenases, superoxide anion, hydroxyl radical, peroxynitrate, haem proteins, ceruloplasmin and myeloperoxidase (Ylä-Herttuala, 1994). LDL particles with oxidative modifications of both its protein and lipid moieties are known to exist in atherosclerotic lesions and are readily internalized by macrophages. A number of cell membrane proteins that can bind oxidized LDL with high affinity have been identified on the surface of macrophages, endothelial cells and smooth muscle cells. One characteristic that almost all of these 'scavenger receptors' (SRs) share is the ability to bind with high affinity to a broad spectrum of structurally unrelated ligands. They are divided to different groups: SR class A consists of SR-AI, SR-AII, SR-AIII, and the macrophage receptor with collagenous structure (MARCO); class B consists of SR-B1 and CD36. The distinct, but partly overlapping, binding properties of the SR classes form a complication in defining their respective activity in terms of ligand uptake. Most SRs bind a variety of polyanionic ligands. SR classes A and B are expressed in atherosclerotic plaques and are involved in the development of lipid-laden foam cells (Steinbrecher, 1999). All the major cell types in atherosclerotic lesions, monocytes, macrophages and SMCs, can modify LDL to a form that can be internalized.

Oxidized LDL has several atherogenic properties. It is metabolized through scavenger receptor, and it causes cellular lipid accumulation (Henriksen et al., 1983) and is itself chemotactic agent for circulating monocytes and can therefore contribute to the recruitment of monocytes into the site of developing arterial lesion (Quinn et al., 1987). Minimally oxidized LDL can also stimulate the release of monocyte chemotactic protein - 1 (MCP-1) from endothelial cells (Cushing et al., 1990) and induce the adhesion of monocytes on endothelium by induction of P-selectin (Vora et al., 1997). In addition, oxLDL has the potential of releasing macrophage colony stimulating factor (M-CSF) from endothelial cells, which can lead to expansion of the macrophage population in a developing lesion (Rajavashisth et al., 1990). OxLDL is cytotoxic and accounts for some of the inflammatory processes occurring during the history of lesion. Multiple lipid oxidation products in oxLDL can induce cytokine production, for example IL-1β, which has been shown to induce SMC

proliferation and is found in atherosclerotic lesions (Thomas et al., 1994).

Evidence supporting the oxidative modification hypothesis has accumulated rapidly in the past several years and there are number of lines of evidence indicating that oxidative modification does occur in vivo and that prevention of it slows the progression of atherosclerotic lesions (Witztum and Steinberg, 1991; Carew et al., 1987).

2.2.3. Oxidized phospholipids and PAF

The average LDL particle contains 1 molecule of apolipoprotein B (apoB), 600 molecules of free cholesterol, 1600 molecules of cholesteryl ester, 700 molecules of phospholipids, 180 molecules of triacylglycerol and approximately 10 molecules of alpha-tocopherol (α-TOH), the major endogenous antioxidant (Hevonoja et al., 2000). All these components can be subjected to cell-mediated oxidation. Phospholipids compose the outer monolayer of lipoproteins and the membranes of lesion cells. In lipoproteins, the phospholipid monolayer provides an amphipathic interface between the neutral lipid core and the aqueous external environment. In atherosclerosis, the phospholipids of lesion lipoproteins are modified by various oxidative reactions that have important pathological consequences. The polyunsaturated fatty acid groups (mainly arachidonate and linoelate) of phospholipids and triacylglycerols are oxidized to hydroperoxides. Oxidation of phospholipids results in chain-shortened fragments and oxygenated derivates of polyunsaturated sn-2 fatty acyl residues, generating a myriad of phospholipid products. A typical mass-spectometric analysis of intact glycerophospholipids derived from oxidized LDL suggests that hundreds of radical products results (Marathe et al., 2000). One type of phospholipid oxidation product mimics the structure of the potent inflammatory mediator platelet-activating factor (PAF), containing a shortened sn-2 acyl chain and an ether-linked fatty acyl group in the sn-1 position, and these oxidation products activate the PAF receptor found on platelets, monocytes and leukocytes (Heery et al., 1995). Production of such PAF mimetics is, in contrast to the physiologic generation of PAF, uncontrolled (Tokumura et al., 1987). PAF mimetics and other phospholipid oxidation products are found in atherosclerotic lesions or even in blood after exposure to cigarette smoke (Lehr et al., 1997; Watson et al., 1997).

Platelet activating factor (PAF; 1-alkyl-2-acetyl-sn-glysero-3-phophocholine), and PAF-like lipids, are biologically active phospholipids and they posses many atherogenic activities. The inflammatory actions of PAF include platelet aggregation, hypotension, anaphylactic shock and increased vascular permeability (Prescott et al., 2000). Monocyte and SMC activation is

atherogenic, and PAF and its mimetics activate monocyte cytokine secretion (Weyrich et al., 1995) and SMC growth (Heery et al., 1995)- both components of inflammation in atherogenesis and vascular remodelling. In addition, oxidized phospholipids represent one class of ligands on oxLDL that mediates its binding and uptake by macrophage scavenger receptors (Horkko et al., 1999). The possible involvement of PAF in cholesterol deposition in the arterial wall has been investigated in rabbits fed a hypercholesterolemic diet. The oral administration of a PAF receptor antagonist to these rabbits significantly reduced the amount of esterified cholesterol in the aorta without affecting the plasma levels of cholesterol (Feliste et al., 1989). Clinical studies show higher levels of PAF in coronary artery samples from patients with severe atherosclerosis (Mueller et al., 1995). PAF and PAF-like lipids are inactivated by PAF-acetylhydrolase (PAF-AH), also called as lipoprotein-associated phospholipase A_2, an enzyme which circulates in the blood bound to lipoproteins and catalyzes the hydrolysis of the acyl group at the sn-2 position rendering PAF inactive (Stafforini et al., 1997) (figure 1). Serum enzymes other than plasma PAF-AH have been shown to degrade PAF: lecithin:cholesterol acyltransferase (Liu and Subbaiah, 1994) and paraoxonase (Rodrigo et al., 2001). The table 1 summarizes the biological functions of PAF in cardiovascular system.

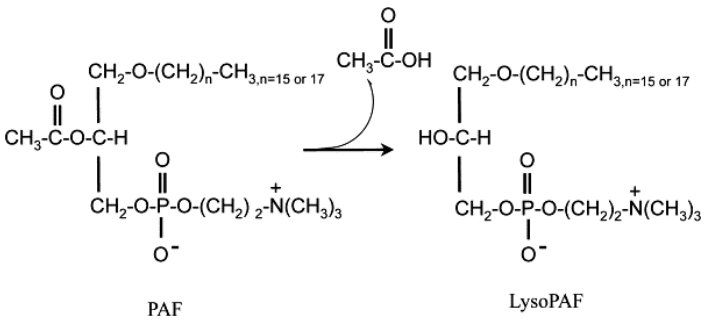

Figure 1. Degradation of PAF by PAF-AH.

A. Hemodynamics
hypotension
platelet activation and aggregation
bradycardia
B. Inflammation
stimulation of neutrophil chemotaxis and vascular permeability
leukocyte adhesion to endothelial cells
activation of monocytes and macrophages
superoxide anion production
cytokine production (IL-1, TNF-α, IL-6, IL-8)

Table 1. Biological functions of PAF (Montrucchio et al., 2000; Zimmerman et al., 2002).

2.2.4. Inflammation

Oxidation of LDLs is an early and causal step in atherogenesis that generates inflammatory compounds leading to foam cell formation. One class of oxidatively generated inflammatory compounds previously discussed are phospholipids that structurally mimic PAF, the PAF-like lipids, which interact and activate the PAF receptor and cause stimulation of platelet aggregation, leukocyte activation and adhesion to endothelial cells, increase vascular permeability and activate monocytes and macrophages to produce superoxide anion (Smiley et al., 1991).

Activation of the endothelium of the arterial intima is characterized by induction of oxidative stress, elevated endothelial permeability and expression of adhesion proteins for inflammatory cells (Williams and Tabas, 1995; Lusis, 2000). Inflammatory cells, including monocytes, macrophages and lymphocytes (predominantly T cells), are critical to the development of atherosclerosis (Ross, 1999). Circulating monocytes and lymphocytes are attached to arterial sites of endothelial injury by the stimulation of chemoattractant cytokines (chemokines), a large and complex family of small proteins that signal through specific chemokine receptors on the surface of inflammatory cells (Gerszten et al., 2000). Interleukin-6 (IL-6) is the principal procoagulant cytokine. It can increase plasma concentrations of fibrinogen, plasminogen activator inhibitor type 1 (Libby and Simon, 2001), and CRP, which amplify inflammatory and procoagulant responses (Pasceri et al., 2000). Inflammatory cytokines, including IL-1, tumor necrosis factor (TNF) and CRP induce the expression of cellular adhesion molecules (Willerson, 2002).

Endothelial adhesion molecules-including E-selectin, intercellular adhesion molecules and vascular cell adhesion molecule-1-have specific roles leading to rolling, firm adhesion and ultimate entry of these cells into the arterial wall (Springer, 1994). The injury also induces the endothelium to have procoagulant instead of anticoagulant properties and to form vasoactive growth factors. Continued inflammation results in increased numbers of macrophages and lymphocytes which both emigrate from the blood and multiply within the lesion. Activation of these cells leads to the release of hydrolytic enzymes, cytokines, chemokines, and growth factors (Libby et al., 1995; Raines et al., 1996), which can induce further damage and eventually lead to the focal necrosis (Moreno et al., 1994).

Inflammatory changes in the arterial wall have a central role also in the development of restenosis. It has been shown that coronary angioplasty provokes an acute inflammatory response; it increases neutrophil, monocyte and platelet adhesion molecule expression (Serrano, Jr. et al., 1997). After angioplasty, monocytes and macrophages appear within the atheromatous plaque along with circulating leukocytes and platelets, which adhere to the angioplasty site and produce cytokines, particularly IL-1 and -6 which in turn trigger the liver to produce inflammatory proteins (Pietersma et al., 1995). Numerous studies have reported a positive association between several systemic inflammatory markers and restenosis. These markers include C-reactive protein, serum amyloid A and fibrinogen, the same markers that are associated with the development of atherosclerosis (Schillinger et al., 2002). Furthermore, a strong link between the presence of leukocytes and the extent of restenosis has been reported in both animal models and humans (Kornowski et al., 1998; Farb et al., 2002). However, pure mechanical trauma from the angioplasty balloon that results in plaque rupture may not necessarily be the culprit for the enhanced inflammatory response but rather may be related to the existing degree of inflammation.

2.3. Restenosis

The nonsurgical treatment of coronary artery disease began with the introduction of balloon angioplasty (PTCA) in the late 1970's. The major drawback of PTCA was recurrence in the first 6-12 months following the procedure, called restenosis (recurrent narrowing). Although the advent of coronary stent has reduced the incidence of restenosis, the in-stent stenosis rate is still 15-20% for ideal coronary lesions, and may increase to over 30-60% for patients with complex lesions (e.g. small vessel, diffuse and bifurcation lesions) (Fattori and Piva, 2003). Restenosis is an obstruction of a blood vessel due to growth of tissue at the site of angioplasty or stent implementation and a repeat procedure may need to be performed (Bittl, 1996).

Restenosis is a multifactorial process, initiated by vessel trauma, and involves mechanisms such as inflammation, stimulation of growth factors and platelet activation (Marmur et al., 1992; Le Breton et al., 1996).

2.3.1. Pathogenesis

Restenosis is a maladaptive response of the artery to injury. This vascular response can be divided into four phases: 1) a mechanical phase (early elastic recoil in response to the mechanical dilation of the vessel), 2) a thrombotic phase, 3) a proliferative phase (neointima formation by proliferation of SMC) and 4) a remodeling phase (differentiation of SMC to a synthetic phenotype and extracellular matrix deposition). Table 2 summarizes the molecular mechanisms involved in the development of restenosis and its regulators. The relative contribution of each of these depends on the type of injury. Within minutes following balloon deflation, the artery undergoes elastic recoil due to contraction of the elastin fibers of the inner and external laminae, causing up to a 40% lumen loss. A thrombotic response triggered by endothelial denudation, and medial dissection due to the mechanical injury of PTCA, lead to platelet adherence and aggregation on the exposed subendothelial surface. Neointimal formation is a complicated process involving the recruitment of inflammatory cells to the site of injury, the migration of VSMC from the media to the intima, and the proliferation of these cells. Growth factors and cytokines are the major stimuli for proliferation of SMCs after the injury. Platelets release platelet derived growth factor, transforming growth factor, epidermal growth factor and thrombin, which stimulate the migration, growth and division of SMCs (Serruys et al., 1988). The dynamic process of SMC migration involves changes in matrix synthesis such as degradation and organization (Strauss et al., 1996). Matrix metalloproteinases, effectors of extracellular matrix degradation (Galis and Khatri, 2002), are upregulated after injury (Strauss et al., 1996) and the degradation of the extracellular matrix allows SMCs to migrate to the intima. The role of inflammation in restenosis is now well acknowledged. Angioplasty causes adhesion of inflammatory cells at the injury site and their migration into the artery wall (Huang et al., 2002); insertion of a foreign body, such as a stent, further increases this inflammatory response. Inoue et al have shown that after PTCA there are substantially increased levels of neutrophils from patients undergoing stent implantation compared with patients undergoing balloon angioplasty alone (Inoue et al., 2000).

Apoptotic SMC death has been documented in numerous animal models of acute vascular injury and in human clinical studies (Han et al., 1995; Isner et al., 1995) but the precise

role of apoptosis in the restenosis process is unclear. Previous angioplasty experiments on rabbit and rat arteries have demonstrated that mechanical injury induces apoptosis in up to 70% of medial SMCs within 30 minutes (Perlman et al., 1997) and over a period of several weeks, a group of surviving medial SMCs migrates towards the arterial lumen to form a neointima (Hanke et al., 1990). Apoptotic cells can release cytokines and this can enhance the proliferative response after traumatic injury.

	Molecular mechanism	**Regulators**
1) Mechanical phase	Elastic recoil	No molecular regulation
2) Thrombogenic phase	Adherence and activation of platelets by expression of vWF and TF	Cytokines, VEGF, NO, thrombin, blood flow
	Recruitment of inflammatory cells: expression of adhesion molecules (P-selectin, GP IIb/IIIa, ICAM) and chemotactic factors (IL-8, MCP-1)	Cytokines (IL-1, IL-6, TNFα) Growth factors (PDGF, thrombin)
3) Proliferative phase	SMC migration and proliferation: production of MMPs and growth factors (PDGF, TGFβ, IGF, FGF, VEGF, thrombin, ATII)	Cytokines (IL-1, IL-6, TNFα, IFNγ) NO
4) Remodeling phase	Remodeling (MMPs) and ECM deposition	Cytokines (IFNγ) Growth factors (PDGF, TGFβ, IGF, VEGF)

Table 2. Molecular mechanisms of restenosis and their regulators. (Donners et al., 2003)

2.3.2. Prevention and treatment

The biological processes in pathogenesis of restenosis suggest a number of targets for pharmacological intervention. These therapies can be divided into categories based on mechanisms of action: namely, anti-thrombotic, anti-inflammatory, anti-mitotic, and pro-mitotic agents for targeting of unwanted SMC proliferation or desirable endothelial cell re-growth, respectively. Traditional pharmacological agents including antiplatelet agents, anticoagulants, angiotensin-converting enzyme inhibitors, calcium channel blockers and lipid-lowering agents have failed to reduce restenosis rates in clinical studies (Dangas and Fuster, 1996; Popma et al., 1991) mainly because the concentrations required for effective action at the site of injury have not been achieved. There are many cases where a positive animal study correlates with negative clinical trial outcome because the differences in doses tolerated between animal and human (Sarembock et al., 1991; Serruys et al., 1995; Gellman et al., 1991). Probucol is the first pharmacological agent showing to reduce coronary restenosis after angioplasty and the mechanism of preventing restenosis appears

to be independent of its lipid-lowering effect (Lee et al., 1996; Tardif et al., 1997). The positive results obtained with probucol suggest that the restenosis process is associated with oxidative stress. Reactive oxygen species are produced after angioplasty and the generation of reactive oxygen species and oxidation of lipids impairs endothelial function. Oxidative stress exerts toxic effects on vascular SMCs which leads to the activation of inflammatory reactions (Rao and Berk, 1992). Antiapoptic caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp(Ome)-fluoromethylketone (ZVAD-fmk) has been shown to inhibit SMC apoptosis after balloon injury and correspond to a significant reduction in neointimal proliferation (Beohar et al., 2004).

About ten years ago, intracoronary stents became widely available, and reduced the restenosis rate to about 15-30% (Fischman et al., 1994; Serruys et al., 1994). The stent is thin slotted metal tube which is implanted in the coronary artery using a small balloon, in a procedure very similar to standard PTCA. While restenosis can often be easily treated non surgically with the use of balloons, atherectomy devices, and the use of intracoronary radiation therapy (French and Faxon, 2002), the prevention of restenosis remains a highly desirable goal. Recent technology has created a method of "coating" stents; using a coronary stent for local delivery of drugs combines scaffolding with targeted drug action. The initial research and clinical trials have been concentrated on sirolimus (rapamycin), a macrolide antibiotic with immunosuppressive and antimitotic properties (Simonton et al., 1998). Stents are coated with a polymer containing low dose sirolimus then a layer of drug-free polymer, which serves as a barrier to diffusion. While stents may almost eliminate elastic recoil and negative remodelling, they can induce a more pronounced vascular response than angioplasty alone (Edelman and Rogers, 1998). Also, after stent implantation restenosis occurs because of the formation of soft scar tissue in the center of the stent, which blocks coronary blood flow. With the increased usage of the stents, there are reports of problems, such as late stent malapposition, subacute and late thromboses and aneurysm formations due to the toxicity associated with this method of treatment. In addition, the long term effects of stents are still unknown.

Gamma- or beta radiation (brachytherapy) has been proposed as a potential way of reducing restenosis, especially in-stent restenosis, because it is well known that low-dose radiation is highly effective and safe for preventing keloids and treating benign vascular malformations (Waksman, 2001). Also, low-dose radiation can delay normal wound healing and impair SMC function. A number of clinical trials have been completed examining the use of intravascular radiation to prevent restenosis; the most positive results

have come from treatment of in-stent restenosis (Leon et al., 2001; Popma et al., 2002). The suggested beneficial effect of brachytherapy is the inhibition of SMC proliferation and the favorable arterial remodeling. Although brachytherapy is technically simple, it posses several difficulties concerning safety issues and side effects. A well documented consequence of brachytherapy is the aneurismal dilatation of the arterial wall and subacute and late stent thrombosis (Waksman et al., 2000).

It is most important to note that all of these exciting developments only allow us to buy time for an individual. Patients requiring any of these treatments, or bypass surgery, must aggressively work to change their life style; losing weight, ceasing all tobacco use, altering their diets to keep total and LDL cholesterol levels at their lowest possible levels, reducing elevated blood pressure, and if necessary, deftly managing diabetes.

2.4. Vein-graft disease

Surgical bypass and angioplasty are the primary interventional therapies of coronary artery disease but they are limited by the problems of restenosis and graft occlusions. Vein grafts fail due to early thrombosis or due to intimal hyperplasia, which develops as a consequence of early vein graft injury (Davies and Hagen, 1995). It is estimated that during the first year after coronary bypass surgery, between 10 and 15% of venous grafts occlude (Fitzgibbon et al., 1996). In epidemiological studies to investigate risk factors for the development of vein graft atherosclerosis, statistically significant differences were found in the blood cholesterol profile; elevated levels of apolipoprotein B and low levels of high density lipoproteins (HDL) were found to be predictive factors for the development of vein graft disease (Campeau et al., 1984). An increased plasma level of lipoprotein-a, Lp(a), is correlated with stenosis in saphenous vein grafts (Cushing et al., 1989).

2.4.1. Pathogenesis

The success of coronary artery bypass grafting (CABG) although the standard for the treatment of multivessel coronary artery disease, is limited by poor long-term vein graft patency (Motwani and Topol, 1998). Early vein graft thrombosis (within 1 month) occurs up to 15% vein grafts due to graft spasm or technical error (Bourassa et al., 1982), and late vein graft failure occurs as a consequence of early neointimal hyperplasia with later superimposed atheroma, so called vein graft disease (Motwani and Topol, 1998).

Although the pathophysiology of vein graft failure is incompletely understood, numerous relevant molecular targets have been elucidated. The principal modes of injury relate to the adaptive responses of the conduit to the arterial circulation and the activation of inflammatory and coagulation pathways. The

vascular responses to these insults include media SMC proliferation and migration, neointimal hyperplasia and deposition of ECM (Davies and Hagen, 1995). In porcine saphenous vein bypass grafts, in the first week after grafting, adventitial, medial and neointimal thickening occurs as a consequence of increased shear stress, surgical preparative injury and the activation of multiple growth factor and cytokine cascades. This is associated with the infiltration of inflammatory cells, medial SMC proliferation and migration to form a neointima (Angelini et al., 1990). Adventitial myofibroblast proliferation and ECM deposition also results in the formation of a thick neoadventitia (Shi et al., 1996). These myofibroblasts migrate through all the layers of the vessel wall, where subsequent ECM deposition contributes to overall wall thickening. After the first week, wall thickening in porcine vein grafts occurs largely due to the ECM deposition (fibrosis) and neointimal SMC proliferation, however this thickening plateaus after one month (Shi et al., 1996). Matrix metalloproteinases (MMPs) are central to the turnover of the ECM, altering the cell-cell interactions, modifying the extracellular milieu and permitting the movement and division of cells. Increased MMP production, with ECM degradation is a feature of the infiltration of the inflammatory cells as well as the migration of SMCs and myofibroblast (Goetzl et al., 1996).

Surgical preparative injury is known to result in significant endothelial loss in vein grafts, exposing subendothelial matrix and leading to the adhesion of platelets and proteins, such as fibrinogen. Platelet aggregates and mural thrombus may form an occlusive thrombosis and adhesion of neutrophils and monocytes leads to release of a range of factors that can stimulate SMC proliferation and migration (Conte et al., 2002). With the possible exception of in situ grafting, all vein grafts undergo a period of ischemia following reperfusion. This leads to the local generation of superoxide and other reactive oxygen species within the wall, triggering secondary inflammatory cascades and direct cytotoxicity to resident ECs and SMCs (West et al., 2001a). Complement activation, leukocyte recruitment, and endothelial loss are known consequences of reperfusion injury. Surgical vein grafting elicits a complex series of events in the vein wall that begins immediately on implantation. A number of pathophysiologic processes with overlapping timelines have been described which are presented in figure 2.

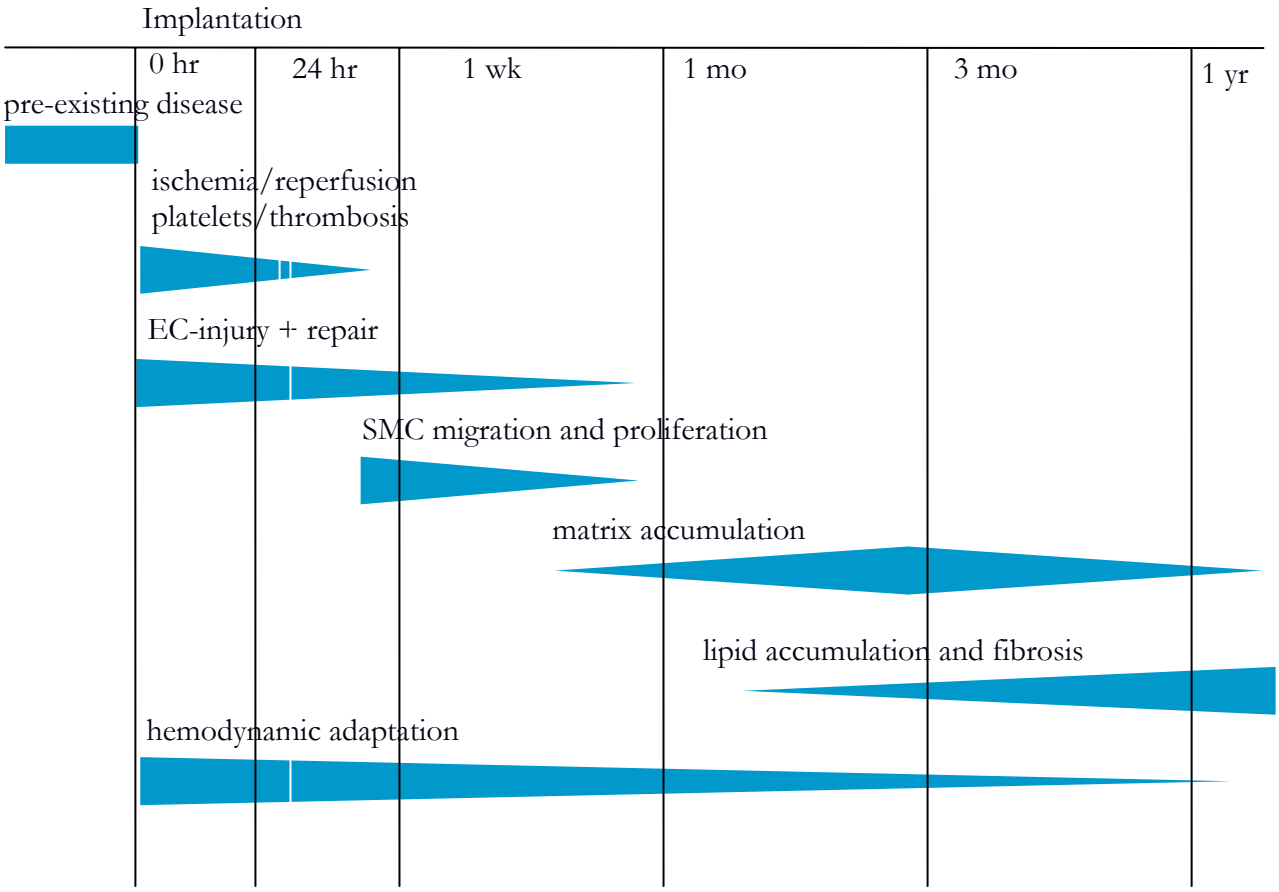

Figure 2. Pathophysiologic events in human vein bypass grafts and their temporal relationships (Conte et al., 2002).

The lesions in vein grafts are usually concentric, involving 90-100% of the circumference of the graft, and diffuse. Foam cells are present on the luminal surface and most of the lesions contain inflammatory infiltrate in the intima, consisting of lymphocytes, plasma cells and macrophages. The grafts are fragile and, in contrast to arterial atherosclerotic lesions, there are no fibrous caps present to prevent the lesion components from contact with the blood stream (Ratliff and Myles, 1989).

2.4.2. Treatment

Vein graft disease differs from arterial atherosclerosis in that its natural history is much shorter and the date of onset is clearly defined, i.e. graft implantation. This process is therefore potentially amenable to strategies that inhibit its progression.

The ability to manipulate vein grafts ex vivo prior to implantation using pharmacological or other methods that may inhibit subsequent disease is a feature unique to vein graft

disease. There are many examples of this being achieved in experimental models, although no systemic agent has yet proven effective in humans. Pre-treatment with rapamycin (Schachner et al., 2004), paclitaxel (Masaki et al., 2004) and the intracellular calcium dependant ATPase inhibitor, thapsigargin (Angelini and Jeremy, 2002), have been shown to significantly inhibit the progression of vein graft disease in experimental models in vivo. Rapamycin is an immunosuppressive agent which also exhibits marked antiproliferative properties (Marx and Marks, 2001). Paclitaxel also is an antiproliferative agent that is effective against normal SMCs and stabilizes microtubules inhibiting mitosis and migration (Grube et al., 2003). Oral agents, such as NO donating aspirins (Shukla et al., 2003) and endothelin antagonists have also been shown to be effective in porcine vein grafts in vivo (Wan et al., 2004) as has the application of a porous external polyester stent which inhibits neointima formation and promotes expansive remodelling in the absence of vein wall thickening (Izzat et al., 1996).

Antiplatelet therapy and anticoagulants have been administered postoperatively based on the assumption that accumulation of platelets and clotting factors plays a significant role in the development of early and late thrombosis and may play an additional role in the development of intimal thickening. In saphenous vein graft bypass, aspirin is recommended 6 hours after surgery. In several clinical series, aspirin and dipyridamole have both been effective in reducing coronary bypass graft failure when administered within 12-24 h after surgery (Clowes, 1986).

3-hydroxy-3-methylgluatryl (HMG-CoA) coenzyme A reductase inhibitors (statins) are a class of cholesterol-lowering drugs that are used extensively for the prevention and treatment of atherosclerosis. The clinical benefits of statin therapy appear greater than would be expected from a simple lowering of cholesterol levels, and these additional effects have been claimed to be due to direct modulation of SMC function; it inhibits SMC proliferation and invasion through a matrix barrier (Bellosta et al., 1998; Porter and Turner, 2002). Simvastatin has been shown to reduce the development of vein graft occlusions in a relatively small cohort of patients (Christenson, 2001).

Angelini et al have investigated the role of external stents on the formation of neointimal hyperplasia in vein grafts. They placed a highly porous, non-restrictive, external polyester stent on saphenous vein grafts that were interposed into the carotid arteries of pigs. Four weeks after implantation, the external stenting resulted in almost four-fold thinner neointima, larger lumen and thinner medial layer when compared with unstented grafts (Angelini et al., 1996). External stenting reduces the expression of PDGF-BB

in vein grafts, indicating a possible mechanism through which external stenting is acting (Mehta et al., 1998).

2.5 Gene therapy for restenosis and vein-graft disease

Recent progress in molecular and cellular biology has developed numerous effective cardiovascular drugs. However, there are still number of diseases for which no known effective therapy exists, such as peripheral arterial disease, restenosis after angioplasty, vascular bypass graft occlusion and transplant coronary vasculopathy. Currently, gene therapy is emerging as a potential strategy for the treatment of cardiovascular disease despite of its limitations. Gene therapy has advantages as compared to the classical pharmacological approach. First, it has the potential to maintain the optimally high and local concentration of therapeutic products over time. In the case of therapeutic angiogenesis, it may be preferable to deliver a lower dose over a period of several days rather than a single or multiple bolus doses of recombinant protein, to avoid side-effects. Secondly, the feasibility of a clinical trial of recombinant protein is currently limited by the lack of approved or available quantities of human quality grade of recombinant proteins. In contrast, gene therapy also has the disadvantages, such as safety aspects and localized and limited effects (Rutanen et al., 2001).

Blood vessels are a very well suited target for gene transfer because they are accessible for minimally invasive, easily monitored catheter-directed delivery during endovascular interventions and directly accessible also during surgery. In many disorders, such as restenosis and vein-graft anastomosis, only temporary expression of the transfected gene will be required to achieve a beneficial biological effect. Medical therapies have had limited impact in reducing vein graft failure or restenosis, so new therapeutic approaches remain of great clinical importance. Gene therapy has an important application in enabling therapeutic concentrations of a gene product to be accumulated at the target site of action. It also offers the possibility of minimizing systemic side effects by avoiding high plasma levels of the gene product. Gene transfer approaches using adenovirus have shown great promise for the therapy of vascular disease (Janssens, 2003; Rutanen et al., 2001).

Restenosis, both in the absence and in the presence of stents, is primarily due to SMC accumulation. Therefore there are many gene therapy approaches to prevent SMC proliferation and migration. Antiproliferative approaches attempt to inhibit cell cycle entry or to cause the death of cells that enter cell cycle. The thymidine kinase isozyme derived form herpes simplex virus is able to phosphorylate and activate ganciclovir that interrupts DNA synthesis. Adenovirus

mediated gene transfer of ganciclovir have been shown to reduce SMC proliferation and neointimal expansion in the rat carotid artery injury model (Chang et al., 1995). It has been shown that downregulation of cyclin G1 expression by retrovirus-mediated antisense gene transfer inhibits SMC proliferation and neointima formation in a rat carotid injury model of restenosis (Zhu et al., 1997). The progress of SMC proliferation is dependent on the coordinated activation of a series of cell cycle regulatory genes that results in mitosis. Therefore, inhibition of the cell cycle using non-phosphorylated retinoblastoma (Rb) gene or anti-oncogenes such as p53 and p21 has been reported in several animal models (Scheinman et al., 1999; Yang et al., 1996b).

The expression of factors promoting endothelial growth may accelerate the recovery of vascular physiology after mechanical injury. This, in turn is potentially associated with reduction in the thrombotic and proliferative environment of the vascular wall. VEGF accelerates endothelial coverage and diminish medial proliferation (Isner et al., 1996). Recently the clinical safety of VEGF gene transfer with plasmid liposome or adenovirus has been demonstrated (Hedman et al., 2003).

Recent progress in molecular biology has provided new techniques to inhibit target gene expression. Especially, the application of antisense strategy to regulate the transcription of disease-related genes in vivo has important therapeutic potential. Antisense oligonucleotides (ODN) are widely used as inhibitors of specific gene expression. The effectiveness of antisense ODN against a proto-oncogene, c-myb, was first reported for the treatment of restenosis (Simons et al., 1992). Recently, the results from a phase II trial using antisense c-myc to treat restenosis has been reported without effect on neointima formation mainly because the single administration of AS-ODN employed in the present trial may not have been effective (Kutryk et al., 2002).

Although a number of studies address the molecular and cellular events that accompany arterial gene transfer, comparatively little is known about gene transfer to vein grafts. The ability to selectively manipulate in vivo the expression of a specific gene within the graft, either by inhibition or overexpression, is a powerful tool in the development of novel therapies. Moreover, the ability to transfer therapeutic genes into the vein wall ex vivo and prior to the onset of the pathophysiologic events that lead to graft disease provides a unique opportunity to alter the pathogenesis of vein graft failure.

At present, adenoviral vectors are the only agents capable of high-efficiency gene delivery to most cells in the vein graft wall within an intraoperative time frame of exposure. In the controlled ex vivo setting of vein graft treatment, the risks of

dissemination or toxicity from systemic exposure appear minimal. Secreted or diffusible mediators with antiproliferative properties are particularly attractive because gene transfer efficiency limitations are lessened by the potential local effect on neighboring cells. All three of the NOS isoforms (endothelial, neuronal and inducible) have been investigated for therapeutic gene transfer to vein grafts. Significant inhibition of graft neointima formation has been reported (range 30% to 50%). The antiproliferative effects of NO are complemented by its additional vasculoprotective properties (antimigratory, reduced platelet aggregation and leukocyte adhesion, vasodilation), making it a proper target (Matsumoto et al., 1998; Shears et al., 1998; West et al., 2001b). Transfection of antisense ODN against PCNA (proliferative cell nuclear antigen) and cdc2 kinase resulted in the inhibition of hyperplasia at 2 weeks after transfection in murine heterotopic cardiac allograft vein graft model (Suzuki et al., 1997). Introducing a synthetic double-stranded (ds) DNA with high affinity for a target transcription factor into target cell as a "decoy" cis element can alter gene transcription (Morishita et al., 1998). The process of SMC proliferation is dependent on the coordinated activation of a series of cell-cycle regulatory genes, which results in mitosis. A critical element of cell cycle progression regulation involves the complex formed by E2F, cyclin A and cdk2. Transfection of E2F decoy ODN into rat balloon-injured carotid arteries and porcine coronary arteries resulted in almost complete inhibition of neointimal formation after balloon injury (Morishita et al., 1995). In addition, clinical trial to treat neointimal hyperplasia in vein bypass grafts with decoy against E2F demonstrated the safety and biologic efficacy of intraoperative transfection of human bypass vein grafts. Phase I/II studies conducted in lower extremity and coronary bypass patients have demonstrated safety and feasibility, and have also suggested possible efficacy. However, large, randomized multicenter, phase III trials failed to show a benefit in the E2F treated group over the 12 months following surgery in peripheral bypass grafts (Mann et al., 1999; Mann and Conte, 2003).

As a result of the investigation that demonstrate an increase in MMPs in vein graft segments (George et al., 1997), studies were performed to determine if the overexpression of tissue inhibitors of MMPs (TIMPs) would reduce neointimal formation in vein grafts. Adenoviral mediated human TIMP-3 gene transfer resulted in evidence of high expression on the lumen and upper ECM of vein segments and this expression inhibits MMP activity and reduces neointimal formation by 58% at four weeks time point (George et al., 2000). Gene transfer of a soluble vascular cell adhesion molecule

(sVCAM) has also been suggested to block monocyte binding to the vascular endothelium through competitive inhibition of binding to the wild-type, cell surface associated VCAM (Chen et al., 1994).

To treat restenosis and vein graft stenosis, we have to have more comprehensive understanding of vascular biology and gene expression and that helps to select the potentially therapeutic gene to be expressed in the vascular wall. We also have to have a vector, which must be capable of efficient gene transfer with an appropriate safety profile. And last, we have to have a range of cathether-based and other approaches to the mechanical delivery of vectors to the target cells. Table 3 summarizes the features of an ideal gene transfer vector.

Highly efficient gene delivery to target cell population with limited transduction of non-target cells
Minimal toxicity and immunogenity
Longevity of transgene expression applicable to the disease
Regulatable transgene expression

Table 3. An ideal gene transfer vector.

2.5.1. Gene transfer vectors

There are many available methods that can be utilised to deliver genes into cells and these can be divided into two main categories, non-viral and viral. Non viral methods include DNA plasmids (naked DNA) and DNA complexes with lipids and cationic polymers. Encapsulation of DNA with lipids or polymers have been studied to protect DNA from degradation and enhance the transfection efficiency (Godbey and Mikos, 2001). Liposomes are chemically engineered particles of bilayer, which spontaneously form a lipophilic coat around DNA plasmids (Hedin and Wahlberg, 1997). Liposome-gene complexes enter the target cell by fusing with the plasma membrane. Polymers bear groups that are protonated at physiological pH leading to the electrostatic attraction between the cationic charge on the polymer and the negatively charged DNA. Cationic polymers have greater versatility in terms of the molecular weight, polymer type, polymer-DNA ratio, molecular architecture and the ability to introduce target-specific moieties. These polycation vectors are internalized into a cell by an endocytotic pathway (Cho et al., 2003) Nonviral methods have some advantages over the viral methods, for example they have low immunogenity and production is easy, but because of the poor uptake of non-viral gene transfer vectors and the limited time expression viral mediated

gene transfer is preferred (Rutanen et al. 2002).

As intracellular parasites, viruses have evolved mechanisms to deliver and express their genes using host-cell machinery. To develop viral vectors, wild-type viruses are engineered to express the gene of interest and modified to eliminate their ability to replicate as wild type viruses in the host. Viral methods of gene delivery including retroviruses, adenoviruses, herpes viruses, lentiviruses and parvoviruses have now been developed.

Recombinant adenoviruses are currently the most efficient delivery system in which high level transient gene expression can be achieved in vivo (Laitinen et al., 1998). Replication defective adenoviruses have a number of advantages over other systems such as retroviruses. These include the ability to infect both dividing and non-dividing cells, the lack of viral DNA integration into the host genome resulting in transient recombinant gene expression, and the ease at production of high titer viral stocks. The majority of cells in the vein graft are non-dividing at the time of gene delivery, so high level infection can be achieved with adenoviruses which are able to infect both dividing and non-dividing cells. Additionally, many studies have also shown the relative ease in which both SMCs and endothelial cells can be infected with recombinant adenoviruses in vitro and in vivo (Rome et al., 1994). However, adenovirus vectors are associated with a local inflammatory reaction that eventually extinguishes transgene expression. Also, circulating antiadenoviral antibodies greatly reduce duration and magnitude of expression (Yang et al., 1996a)

Retroviruses are small RNA viruses and the murine leukemia virus (MMulV) is most commonly used. A potentially attractive feature of retroviral vectors is their ability to integrate the genome of target cells resulting in a long term transgene expression. In the vasculature one cannot expect a highly efficient gene transfer because retroviruses can only transduce dividing cells and in vasculature the mitotic rate is quite low. In the context of restenosis and vein-graft pathogenesis long term transgene expression is not even needed, because the key pathological events happen early after injury. There are still many studies where retroviruses have been used both in preclinical and clinical studies of cardiovascular diseases (Laitinen et al., 1997; Pakkanen et al., 1999). Retroviruses are relative safe to use because they do not express viral proteins on gene-transduced cells, leading to low immunogenity. Retroviruses are difficult to concentrate for high titer production because of the fragile envelope and the transfection efficiency is low. Also the random integration of the transgene causes the risk of oncogene activation.

Lentiviruses such as human immunodeficiency virus (HIV) are a subclass of retroviruses. They also are integrating viruses but they have a unique ability to transduce nondividing, terminally differentiated cells like neurons. The difference with the oncoretroviruses lies within its regulatory and accessory proteins. Rev, one of the regulatory proteins, is of crucial importance to viral replication. It functions by competing with the cell's splicing machinery and has also been shown to inhibit splicing of viral mRNAs (Pollard and Malim, 1998). Tat, the other regulatory protein, enhances the transcriptional activity of the LTR 100- to 500-fold (Jones and Peterlin, 1994). Other lentivirus accessory proteins are Nef, Vif, Vpu and Vpr (Lever, 2000) and they have many functions for example in augmentation of viral release, formation of infectious particles and viruse's ability to escape the immune system (Quinonez and Sutton, 2002). The limitations of using lentivirus vectors in clinical trials are today mainly because of the lack of sufficient methods for producing high-titer virus stocks and the safety concerns related to their origin from HIV, despite the engineering of packaging cell lines and deletions of genes required for viral replication (Vigna and Naldini, 2000).

In addition to lentivirus, the adeno-associated virus (AAV) is one of the newest vectors for gene transfer. Like adenoviral vectors, AAV vectors are able to transduce many different cell types like skeletal muscle, SMCs and central nervous system cells and do not need active cell proliferation for transduction. Additionally, these vectors may be less immunogenic and may obtain a more prolonged transgene expression (Flotte and Carter, 1995). The usefulness of AAV vectors in vascular gene delivery has been demonstrated in cultured SMCs and ECs from various species as well as in vivo gene transfer to the arterial wall (Lynch et al., 1997). However, there are some disadvantages associated with the replication of AAV. The packaging capacity is relatively restricted and the large scale production is inefficient. Furthermore, the integration of recombinant AAV into host genome is random or they remain episomal.

Baculoviruses are a diverse group of viruses having a restricted host range limited to insects (Gröner, 1986). Current evidence suggests that baculoviruses also provide an effective tool for in vivo gene transfer. Carotid arteries of NZW rabbits are successfully transduced by recombinant baculoviruses using a collar device. Transient expression in the adventitial cells was observed with an efficacy comparable to adenoviruses (Airenne et al., 2000). The issue that complement represents a primary hurdle for an in vivo application in serum and blood can be experimentally overcome by application of complement-blocking agents,

resulting in the protection of baculovirus vectors (Hoare et al., 2004). Baculovirus vectors have been injected into rodent brain and no inactivation of the vector by the complement system was seen (Sarkis et al., 2000). From a biological safety perspective, recombinant baculoviruses are potential choice for gene transfer experiments because they are inherently unable to replicate in mammalian cells (Kost and Condreay, 1999).

Gene transfer vectors are compared in table 4. Ideally, a vector for gene transfer should be easily produced, available in a concentrated form, tissue-specific, non-toxic and nonimmunogenic, provide long-term expression and allow a broad range of transgene size. Such a vector currently does not exist.

Vector (packaging capacity)	Advantages	Disadvantages
Adenovirus (7.5 kb)	Ability to produce high titers Efficient expression Tropism for multiple cell types Transduces dividing and nondividing cells	Transient episomal expression Immunogenic
AAV (4 kb)	Long-term expression Chromosomal integration Low inflammatory response Transduces dividing and nondividing cells	Limited DNA capacity Difficult production
Retrovirus (8 kb)	Transduces only dividing cells Long-term expression Chromosomal integration Low inflammatory response	Non-specific integration Low titers Low efficiency
Lentivirus (8 kb)	Transduces dividing and nondividing cells Long-term expression	Safety concerns Difficult production Low titers
Baculovirus (> 50 kb)	High capacity Easy to produce in high titers Non-pathogenic to humans	Transient expression Inefficiency in vivo: wild-type vector is complement-sensitive
naked DNA (>20 kb)	Easy to produce Safe	Low efficacy Transient expression

Table.4. Properties of different gene transfer vectors (Rutanen et al., 2002).

2.5.2. Treatment genes

In study II-IV we used different genes to study their effects on restenosis or vein-grafts; lipoprotein-associated phospholipase A_2 (Lp-PLA_2), a 35kDa vaccinia viral protein (35k) and TIMP-1, VEGF-C and A, and EC-SOD (extracellular superoxide dismutase).

Plasma Lp-PLA_2 is associated with circulating LDL particles, and can protect LDL from the effects of oxidation in two ways. Firstly, oxLDL contains oxidatively fragmented phospholipids with PAF-like bioactivity and these inflammatory and mitogenic phospholipids are destroyed by the plasma Lp-PLA_2 (Heery et al., 1995). In view of the potential role of the oxidatively generated PAF-like lipids in atherogenesis, it is important to note that a PAF receptor antagonist was protective in a rabbit model of atherosclerosis (Feliste et al., 1989). Secondly, oxidation of LDL creates particles that are metabolized in macrophages by scavenger receptors (Krieger et al., 1993) which leads to accumulation of intracellular cholesterol and the formation of foam cells. Lp-PLA_2 activity is decreased in subjects at increased risk for coronary artery disease, including patients with insulin-dependent diabetes mellitus, hypertension and smokers (Imaizumi et al., 1995). Lp-PLA_2 has been shown to inhibit apoB modification and conjugated diene formation (Stafforini et al., 1992) and HDL-associated Lp-PLA_2 prevented oxidation of atherogenic lipoproteins and macrophage homing in apoE-/- mice (Theilmeier et al., 2000).

35k is a soluble vaccinia viral protein that binds and inactivates a wide range of CC-chemokines, which are chemoattractants for lymphocytes and monocytes (Bursill et al., 2003). MCP-1 is the most important CC-chemokine that regulates migration and infiltration of monocytes/macrophages and it causes chronic vascular inflammation and induces thrombosis, proliferation and migration of SMCs, angiogenesis and oxidative stress. Previous studies indicate that MCP-1 production from ECs, SMCs, and lesion leukocytes increases in the presence of endothelial dysfunction and atherosclerotic risk factors (Boring et al., 1998). Monocytes have potent procoagulant and proinflammatory functions, which may contribute to the development of vein graft disease and it has been shown that veins briefly exposed to arterial circulation demonstrated an increased monocyte adhesion (Eslami et al., 2001). Adenovirus-mediated gene transfer of 35k has been showed to reduce atherosclerosis by inhibiting macrophage recruitment in atherosclerotic ApoE-knockout mice (Bursill et al., 2004).

Matrix remodelling is considered to be fundamental to initiation of neointimal hyperplasia of vein-grafts allowing SMC migration, and is dependent on activation of MMPs (Zorina and Jaikirshan, 2002).

Expression of MMP-2 and MMP-9 is increased in vein graft segments contributing to intimal thickening (George et al., 1997). Activity of MMPs is regulated by the interactions with TIMPs and TIMP-1 is found to act as an endogenous inhibitor of MMP-2 and MMP-9 (George, 1998). It has been shown that adenovirus-mediated TIMP-1 gene transfer reduces MMP activity in vein graft segments and decreases neointimal formation (George et al., 1998b).

Superoxide anion (O_2^-) and other reactive oxygen species (ROS) play major roles in vascular biology. High levels of ROS contribute to vascular dysfunction and abnormal cell growth including hypertrophy of vascular SMC (Finkel, 2003). EC-SOD has been shown to reduce O_2- mediated macromolecular and cellular damage, therefore suggesting that EC-SOD gene transfer may be used to attenuate tissue damage caused by oxygen-derived free radicals (Strålin et al., 1995). Laukkanen et al also showed that local catheter-mediated delivery of EC-SOD adenoviruses can reduce restenosis in rabbits (Laukkanen et al, 2002). EC-SOD expression is substantially reduced in patients with coronary artery disease, suggesting that reduced EC-SOD activity contributes to endothelial dysfunction (Landmesser et al., 2000)

2.5.3. Combination therapy

In the future, as the pathological processes in arteries are better understood, several therapeutic genes could be combined and these "gene cocktails" are expected to produce enhanced therapeutic effects in vascular gene therapy. Putzer et al showed in murine model of breast cancer that combining immunomodulatory and antiproliferative genes is effective; the combination of Adp53wt (1 x 10^9 pfu) plus a relatively low dose of AdIL-2 (1.5 x 10^8 pfu) caused regressions in 65% of the treated tumors without toxicity (Putzer et al., 1998). There are also few combination therapy studies concerning ischemic myocardium. Combination of plasmids angiopoietin-1 and VEGF-A enhanced arteriogenesis in the ischemic myocardium (Siddiqui et al., 2003) and the combination of plasmids bFGF and PDGF-BB increased the amount of both capillaries and arterioles and in addition gave rise to stable capillaries compared to single factor transfer (Hao et al., 2004).

2.5.4. Clinical trials

Vascular gene therapy is a quite new approach in clinical practice and only limited results are available. Currently, most of them are focused on therapeutic angiogenesis and the treatment of postangioplasty restenosis (Rutanen et al., 2002). Principle reasons for the disappointingly slow clinical implementation of gene therapy for restenosis

are an incomplete understanding of the vascular biology of restenosis, the difficulty of translating findings in animal models into the human setting and the technical difficulties in localized gene delivery into coronary arteries.

Thus far, the major limitation for clinical anti-restenotic gene therapy are concerns about the safety and efficacy of vector systems in use for the local overexpression of transgenes, which in turn is one of the most attractive advantages of gene therapy compared to systemic drug therapy. Hedman et al have shown that intracoronary local VEGF gene transfer given during angioplasty can be performed safely without major gene transfer-related adverse effects after the 6-month follow-up (Hedman et al., 2003). Another example of using the angiogenic factor is the study of adenovirus-mediated intracoronary gene transfer of fibroblast growth factor-4 for patients with myocardial ischemia. Improvements were seen in exercise capacity and myocardial perfusion, but these changes did not reach the statistical significance (Grines et al., 2002). Kutryk et al. studied whether antisense oligodeoxynucleotides (ODN) directed against the nuclear proto-oncogene c-myc could inhibit restenosis when given by local delivery immediately after coronary stent implantation, but no results with reduced neointima volume or restenosis rate were achieved (Kutryk et al., 2002).

Recent studies using decoy oligodeoxynucleotide, which binds and inactivates the pivotal cell-cycle transcription factor E2F have shown that intraoperative transfection of human bypass vein grafts with E2F-decoy is safe, feasible, and can achieve sequence-specific inhibition of cell-cycle gene expression and DNA replication. Twelve months postoperatively, there were fewer patients with graft occlusions or critical stenosis; moreover, fewer patients underwent revision surgery in the E2F decoy group compared with the control group (Mann et al., 1999). However, large-scale phase III trial of E2F decoy in peripheral artery bypass graft failure did not show any benefit. The E2F decoy is being evaluated in phase III trial in coronary artery bypass grafts and results are published later this year (http://www.medicalnewstoday.com).

2.6. Animal models

Studies of restenosis and vein-graft stenosis in humans are limited by the fact that direct tissue examination is only rarely possible. Animal studies allow direct examination of tissue and the ability to vary experimental conditions. However, animal models are not perfect mirrors of human pathology, and there are numerous examples of therapies that are effective in animals but not in humans. Therefore, animal studies are best used to answer specific biological questions that give insights into human disease rather than to

provide exact surrogates of human pathology (Schwartz et al., 2004).

2.6.1. Restenosis

Animal studies have yielded various results on the relative importance of remodelling and neointimal formation in the pathogenesis of restenosis (Post et al., 1994; Lafont et al., 1995). Animal models, however, have different proliferative and thrombogenic response to arterial trauma, and plaque content is often different than what is found in human atherosclerotic stenoses requiring angioplasty. Experimental models do not display the features of complex atheroma, such as calcification, central necrosis, thrombus formation and plaque haemorrhage. Still, animal models are crucial to the development of novel and effective human therapies.

Extensive studies on the response to vascular injury were performed years before the development of angioplasty. These studies were primarily performed in the rat carotid artery, and utilized a very different type of injury. Instead of medial rupture with high-pressure balloons, low-pressure balloon was used to remove cells from the intima. These studies identified the intimal layer as the key site in the proliferative response seen with this type of injury. Unfortunately, clinical trials of agents that prevented the restenotic response to this injury in rats were ineffective in humans (Powell et al., 1989; Hermans et al., 1991). The rat common carotid artery angioplasty model involves inducing fibroproliferative lesions within a long unbranched segment of artery. After denudation of the endothelium, a hyperplastic neointimal response to injury is induced following repeated withdrawal of an inflated balloon catheter. Classically, the response to the injury in this model is referred to as the "three wave paradigm", whereby endothelial denudation produces medial SMC proliferation (peaking 3 days after injury), SMC migration (from the media to the subendothelial/intimal border 4 days following injury), and finally, intimal proliferation coincidental with matrix synthesis (resulting in neointimal formation, peaking 1 to 2 weeks after injury) (Clowes et al., 1983; Lindner and Reidy, 1991). Rat model is far from perfect; although the rat´s size is advantageous over large animals (ease of handling, expense of husbandry), its small body may constitute a disadvantage. A rat has a different dynamic vessel wall shear stress than a human and it causes differences in the repair process. The carotid artery of the rat is an elastic vessel, unlike the human coronary circulation, which is composed of muscular arteries. Thus, this model does not accurately reproduce the vasomotor components observed clinically. These differences are less evident in larger species such as pigs. Also, unlike porcine, canine and non-human primate models, angioplasty-induced platelet

adherence, mural thrombus formation and fibrin deposition are relatively unimportant processes in the rat. This is in contrast to the profound activation of the coagulation system that occurs in humans after PTCA (Ip et al., 1991).

An atherosclerotic rabbit model has also been used to model the arterial injury that occurs in angioplasty. Unlike rats, hypercholesterolemia can be induced readily in rabbits using either atherosclerotic diet or strains of rabbits with a genetic predisposition to developing hypercholesterolemia (Watanabe heritable hyperlipidemic, WHHL, rabbits). A lesion then develops after denudation of the iliac arteries with an angioplasty balloon. Although the lipid profiles obtained in rabbits bear little relationship to those seen in humans, reproducible foam cell-rich lesions can be induced. Even though the restenotic lesions are somewhat dissimilar from human lesions, this model has provided insights into mechanism of repair after injury.

The most widely used and accepted model for studies on restenosis is the coronary overstretch balloon injury model in the pig (Muller et al., 1992). The pig is very similar to humans in its cardiovascular anatomy, physiology and coagulation systems. In this model pigs undergo an angioplasty procedure with the same equipment that is used clinically. The ballooning causes a medial tear that is similar to what is seen clinically. In addition, the neointima formed in response to the balloon injury in the porcine model closely resembles the neointima seen in clinical samples (Schwartz et al., 1992). Despite the limitations of this model, success of an agent in the pig model generally correlates with clinical success.

2.6.2. Vein-graft disease

An essential requirement for the development of gene therapy for vein graft failure is the availability of suitable experimental models, both in vitro and in vivo. A number of in vivo experimental models have been used in the study of vein graft disease, including rabbit, canine, sheep, porcine and non-human primate species (Mehta et al., 1996). Technical challenges associated with coronary artery grafting in small animals has led to models based on end-to-end or end-to-side interposition of vein segments into peripheral arterial sites such as the carotid or iliofemoral arteries. The donor vein is usually external jugular, cephalic, femoral, or the saphenous vein. One of the favored models is the porcine model, in which bilateral saphenous vein-into-carotid artery interposition grafts are performed using end-to-end anastomosis (Angelini et al., 1990). Physiologically, the animal shares similar coagulation profiles and lipoprotein metabolism with humans, and is susceptible to spontaneous and diet-induced atherosclerosis (Mehta et al., 1996). Cell and

organ culture-based studies have confirmed that porcine SMC behave very similarly to their human counterparts.

Cuff technique is feasible in mouse model to obtain reproducible results (Zou et al., 1998). The vessel segment of either vena cava or jugular vein from donor is grafted between two ends of the carotid artery by sleeving the ends of the vein of the recipient mouse over the artery cuff. The simplified mouse model of vein grafts has several advantages: first, the operation procedure is simple and the traumatic and ischemic injuries to the grafts are minimal. After cell death in the early stage of vein grafts, endothelial regeneration and SMC accumulation in the intima occur. It has been shown that regenerated endothelial cells of vein grafts originate from recipient circulation, and not from the remaining endothelial cells of the donor vessels. Circulating progenitor endothelial cells adhere to the grafts and subsequently cover the surface of neointimal and atherosclerotic lesions of vein grafts (Xu et al., 2003).

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Gene transfer of reporter gene has been used in various animal models of vein graft disease. Kupfer et al evaluated β-galactosidase expression 3 and 7 days following transfer in rabbit jugular veins (Kupfer et al., 1994). Channon et al performed adenoviral –mediated β-galactosidase transfer to rabbit jugular veins (Channon et al., 1997) and Chikada optimized adenoviral-mediated β-galactosidase transfer to rabbit jugular vein grafts by the addition of dimethylsulfoxide and hyaluronidase (Chikada and Jones, 1999).

As a model for human vein graft disease, organ culture of human saphenous vein segments is available. In these cultured segments a neointima develops within 4 weeks. This in vitro model vein graft has close morphological resemblance to the very early lesion in human vein grafts in vivo, providing a model for testing specific aspects of human vein graft disease (George et al., 1998a)

3. Aims of the study

The aim of the study was to investigate the roles of four treatment genes in restenosis and vein graft stenosis. More specifically, the following questions were addressed:

1. Can increased Lp-PLA_2 content protect LDL particles against oxidation and prevent foam cell formation in vitro? (study I)

2. Is local adenovirus-mediated gene transfer of Lp-PLA_2 effective in reducing neointima formation after balloon angioplasty in a rabbit model? (study II)

3. Are there differences in the treatment effects between vaccinia virus anti-inflammatory protein 35k and TIMP-1 on vein graft stenosis after adenovirus-mediated gene transfer in a rabbit jugular vein graft model? (study III)

5. Does the combination of the treatment genes (35k, TIMP-1 and EC-SOD) enhance the treatment effect in the rabbit vein graft model? (study IV)

4. Materials and methods

All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. Cell culture reagents were from Gibco BRL unless otherwise stated. Cell lines were from ATCC and chamber slides from LabTek Brand, Nunc International. ^{125} I was from NEN Life Science Products. Apoptosis was detected with an ApopTag kit (Intergen).

4.1. Cell culture studies

4.1.1. LDL isolation and modification

LDL was isolated for in vitro studies from New Zealand White rabbits after the gene transfer. Fentanyl-fluanisone (0.3 ml/kg s.c.; Janssen Pharmaceutica) and midazolam (1.5 mg/kg, i.m.; Roche) were used for anaesthesia. The gene transfer was done via common carotid artery with 5F introducer (Cordis Corporation) after two weeks of 0.5% cholesterol diet. The doses of adenovirus (AdLp-PLA$_2$ and AdLacZ) were 10^8, 10^9 and 10^{10} pfu (plaque forming units). Serum (40-60 ml/rabbit) was collected for LDL isolation and enzyme activity measurements. LDL was ultracentrifugated (Ylä-Herttuala et al., 1989) and radioiodinated with I^{125} using Iodogen (Pierce Chemical Co, Rockford, USA) as an oxidizing agent (Bilheimer et al., 1972) before standardized 18 h incubation with Cu^{2+} (20μM) (Ylä-Herttuala et al., 1989). In one group, Lp-PLA$_2$ activity was irreversibly inhibited by 0.1 mM Pefabloc (4-[2-aminoethyl]-benzenesulfonyl fluoride) (Dentan et al., 1996) at 37ºC for 30 min before LDL iodination and oxidation. Dialysis steps were performed overnight in 0.9% NaCl/0.01% EDTA, pH 7.4, at +4ºC. After incubation the medium was analyzed using agarose gel electrophoresis (Paragon Lipoprotein Electrophoresis kit, Beckman, Namur, Belgium).

4.1.2. In vitro transfection efficiency

RAW 264 (ATCC) cells and rabbit aortic smooth muscle cells RAASMC (Ylä-Herttuala et al., 1995) were transduced with adenoviruses at MOIs (multiplicity of infection) 100, 1000 and 5000. The medium was changed to Optimem containing 0.5% lipoprotein deficient serum (LPDS) and 1% penicillin streptomycin. The cells were grown for 48 h and medium was collected and lyophilised for enzyme activity analysis and immunoblot. Total RNA was isolated for RT-PCR analysis using Trizol Reagent (Gibco-BRL).

4.1.3. Immunoblot and RT-PCR analysis

Lyophilised medium of the AdLp-PLA$_2$ transduced RAW 264 cells and SMCs was subjected to 12% SDS-PAGE. Samples were mixed with loading buffer (50mM Tris-HCl pH 6.8/2% SDS/0.1% bromophenol blue/10% glycerol) and incubated for 4 min at 95°C prior applications into the gel. The resolved

proteins were blotted on Immobilon PVDF membranes (BioRad). Lp-PLA_2 was detected with human PAF-AH polyclonal antiserum according to manufacture's instructions (Cayman Chemical). Human plasma PAF-AH was used as a positive control (Cayman Chemical).

For assessment of Lp-PLA_2 mRNA expression, RT-PCR was performed from liver tissue 7 days after the gene transfer and also from RAW 264 and SMC cells 48 h after virus transduction. Total RNA was isolated from the liver samples and from cell cultures after homogenization in Trizol reagent (Gibco BRL) and treated with RQ1 RNAse-free DNAse (Promega). Four μg of total RNA was reverse-transcribed using random hexamer primers (Promega) and M-Mulv Reverse Transcriptase (New England BioLabs). cDNA was amplified by PCR using DyNAzyme™ II DNA Polymerase (Finnzymes) and primers specific for human Lp-PLA_2 sequence as follows: forward 5'-TGGAGCAACGGTTATTCAG-3' ; reverse 5'-TGGTTGTGTTAATGTTGGTCC-3'. Reaction was subjected to 45 cycles: denaturing at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 1 min. Extension in the final cycle was 7 min.

4.1.4. LDL degradation assay and foam cell formation

Degradation assay is used as an index of receptor-mediated uptake of modified LDL. When cells are incubated with modified, iodine radiolabelled LDL it enters the cells via scavenger receptor and is degraded in lysosomes. The iodine is returned into medium in a different form than free, unbound iodine and they can be separated for the gammacounter measurements. Briefly, RAW 293 cells were incubated in Optimem containing 10% LPDS with 10 μg/ml of oxLDL isolated from rabbits given different adenovirus doses (10^8, 10^9 and 10^{10} pfu Lp-PLA_2 or LacZ adenovirus). LDL from one Lp-PLA_2 group was treated with Lp-PLA_2 inhibitor Pefabloc (0.1 mM). The media were collected at different time points (3, 6, 12 and 24 h). After incubation cells were washed and the amount of ^{125}I-labeled acid-soluble material in the medium (degradation) was determined (Ylä-Herttuala et al., 1989). Values obtained from empty wells were subtracted before calculating the results. Protein concentrations from washed cells and LDL were determined by the method of Lowry (Lowry et al., 1951).

Foam cell formation studies measure the uptake of larger amounts of modified LDL than in the degradation assay. RAW 264 cells were plated on chamber slides and incubated for 18 h with Optimem/10% LPDS containing

100 μg/ml oxLDL (Laukkanen et al., 2000a). LDLs used in the experiment were isolated from rabbits given different adenovirus doses. One Lp-PLA_2 group was treated with Lp-PLA_2 inhibitor Pefabloc. After incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde and stained with oil red O in 60% isopropanol, 0.4% dextrin and hematoxylin.

4.2. Animal models

All studies were approved by the Experimental Animal Committee of the University of Kuopio.

4.2.1. Restenosis

New Zealand White male rabbits were fed 0.25% cholesterol diet for 2 weeks prior the experiments and were randomly divided into study groups. Gene transfer was performed 3 days after the balloon denudation. The whole aorta was denuded twice with a 3.0F arterial embolectomy catheter (Sorin Biomedical). Three days later, the gene transfer was performed with a 3.0F channelled-balloon local drug delivery catheter (Dispatch catheter, Boston Scientific), which allows continuous blood flow during transduction (Laitinen et al., 2000). Under fluoroscopic control, the catheter was positioned caudal to the left renal artery in a segment free of side branches (figure 3). A virus titer of $1.15x10^{10}$ pfu was used in the final volume of 2 ml in 0.9% saline, and the gene transfer was performed at 6 atm pressure for 10 min (0.2 ml/min). Animals were sacrified two or four weeks after the gene transfer. Serum samples were collected before gene transfer and at time points 3, 7, 14, and 28 days after the gene transfer. Tissue samples from liver, spleen, lungs and kidneys were collected to determine the biodistribution of adenovirus.

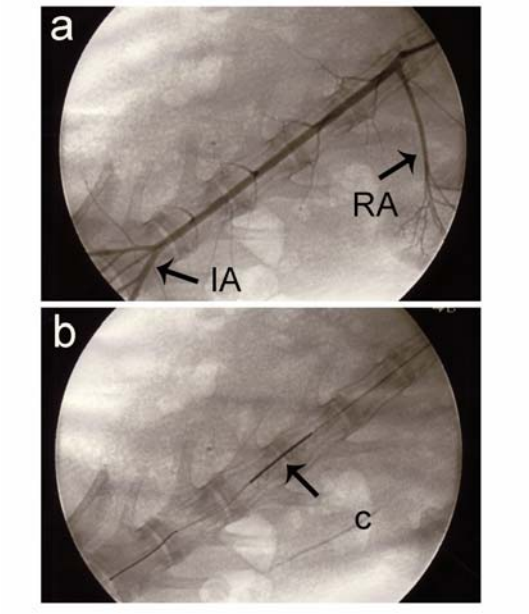

Figure 3. Gene transfer to rabbit aorta using Dispatch-catheter. A: Angiogram from rabbit abdominal aorta. B: Dispatch-catheter in rabbit aorta.
RA= renal artery, IA= iliac artery,
C= dispatch catheter.

4.2.2. Vein-graft

Forty New Zealand White rabbits were randomly divided into study groups for two weeks and four weeks time points (five rabbits in each study group). Virus was used at the titer of 1.0 x 10^9 pfu /ml.

End to side anastomosis was made to the right common carotid artery using the right external jugular vein. The vein was first dissected free from the surrounding tissues and all branches were ligated. The proximal side of the vein was clamped and the bifurcation branches were ligated. A hole was made in the right bifurcation vein right behind the ligation 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 injection of 500µl of the virus. The virus was kept in the vein for 10 min with a slight pressure. Virus was then removed, the vein was flushed with saline and detached right below the bifurcation. No virus was released to the systemic circulation. Carotid artery was dissected free form surrounding tissues 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. After anastomosis was finished the clip form the proximal end of the vein was removed. Clips from the carotid artery were removed and blood flow was established. Rabbits were sacrificed two weeks and four weeks after the surgery and gene transfer.

4.3. Virus vectors

4.3.1. Production of adenoviruses

The Lp-PLA_2 adenoviruses were constructed and produced with the Adeno-X™ Expression System (Clontech), which is based on serotype 5 adenovirus. Human Lp-PLA_2 cDNA (Tew et al., 1996) was cloned into Adeno-X-Viral DNA with CMV immediate early promoter and bovine growth hormone polyA. The recombinant Adeno-X DNA was packaged into infectious adenoviruses by transfecting HEK 293 cells using Fugene 6 reagent (Boehringer-Mannheim). Before preparation of high-titer viral stocks by CsCl gradient centrifugation, the identity of the virus was confirmed from viral DNA by PCR using human Lp-PLA_2 specific primers. The production of LacZ control adenoviruses has been previously described (Laitinen et al., 1998). Purified virus preparation was analyzed for the absence of toxicity, wild-type viruses, microbiological contaminants and lipopolysaccharide as described (Laitinen et al., 1998). Functionality of the AdLp-PLA_2 was verified at mRNA, protein and enzyme activity levels in vitro (Turunen et al., 2004).

TIMP-1, EC-SOD, 35k and lacZ adenoviruses used for the study have been described previously (Laitinen et al., 1998; George et al., 1998b; Bursill et al., 2003;

Laukkanen et al., 2000b). Replication-deficient E1-E3 deleted adenoviruses were produced in 293 cells (Laitinen et al., 1998). 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 (Laitinen et al., 1998; Puumalainen et al., 1998). All viral lots were analyzed for the absence of microbiological contaminants, mycoplasma and lipopolysaccharide (Laitinen et al., 1998; Puumalainen et al., 1998). The TIMP-1, EC-SOD, 35k and lacZ adenoviruses were driven by the CMV promoter and lacZ adenovirus also had a nuclear targeted signal.

4.4. Histology

The histology was evaluated by light microscopy on paraffin embedded sections counter stained with Mayer's Carmalum solution.

For proliferation index measurements, three hours before death animals were injected with 50 mg of bromodeoxyuridine (BrdU) dissolved in 40% ethanol. After death, the transfected segment was removed, flushed gently with saline, and divided into four equal parts. The proximal part was snap-frozen in liquid nitrogen and stored at -70°C. The next part was immersion-fixed in 4% paraformaldehyde/15% sucrose (pH 7.4) for 4h, rinsed in 15% sucrose (pH 7.4) overnight, and embedded in paraffin. The medial part was fixed in 4% paraformaldehyde/PBS (pH 7.4) for 10 min, rinsed in PBS, embedded in OCT compound (Miles), and stored at -70°C. The fourth part for BrdU sections was fixed in 70% ethanol overnight and embedded in paraffin. Tissue samples for biodistribution analysis were collected, flushed with saline and divided into two parts. One part was snap frozen in liquid nitrogen and stored at -70 ºC. The second part was immersion fixed in 4% paraformaldehyde/15% sucrose (pH 7.4) for 4 h, rinsed in 15% sucrose (pH 7.4) overnight and embedded in paraffin.

The avidin-biotin-horseradish peroxidase system (Vector Laboratories, Burlingame, CA, USA) was used in all immunocytochemistry studies.

4.4.1. X-gal stainings and immunohistochemistry

Evaluation of the gene transfer efficiency was done by using x-gal staining of OCT embedded tissue sections (Laitinen et al., 1997). Samples for β-galactosidase assay were fixed in 4% paraformaldehyde/PBS (pH 7.4) for 10 min, rinsed in PBS, embedded in OCT compound (Miles). Cryosections were stained with x-gal reagent, which was prepared by dissolving 1 g 5-bromo-4-chloro-3-indolyl-β-D-galactoside (MBI Fermentas) to 10 ml dimethylformamide. X-gal reagent was diluted 1:100 with x-gal solution (5 mM

$K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$) and pipetted directly on the frozen sections and incubated in dark at 37 ºC for 8 h. Sections were rinsed with 1 x PBS, dehydrated with rising ethanol concentrations, counterstained with Mayer's Carmalum and embedded with Permount (Fischer Scientific).

The paraffin sections were used for immunocytochemistry. Table 5 shows antibodies used in this study.

For control immunostainings the primary antibody was omitted. After immunostaining tissue sections were counter stained with hematoxylin or Mayer's Carmalum counter staining solution.

antibody	target	dilution	producer	article
RAM-11	rabbit macrophages	1:50	DAKO	II-IV
CD-31	endothelial cells	1:50	DAKO	II-IV
HHF-35	SMC α-actin	1:50	DAKO	II-IV
TIMP-1	clone IM63	1:10	Oncogene	III
MMP-2	clone IM33L	1:25	Oncogene	III
MMP-9	clone IM37L	1:25	Oncogene	III
PAF-AH	human PAF-AH	1:500	Cayman	I

Table 5. Antibodies used for immunocytochemistry

4.4.2. Measurement of cell proliferation and morphometry

The proliferation index in the arteries and vein-grafts was determined using the 5'-bromo-2'-deoxyuridine (BrdU) labelling. The labelling index was calculated as the percentage of the BrdU-positive nuclei of the total number of the cells in the section.

Morphometry and image analysis were done by using haematoxylin-eosin stained paraffin sections and analysis of intima/media ratio were performed using Olympus AX70 microscope and analysis software (Soft Imaging Systems, GmbH) (Hiltunen et al., 2000).

4.5. Analysis of plasma samples

Blood samples were collected at time points 0, 3, 7, 14, 21 and 28 days after the gene transfer. Blood plasma values of Lp-PLA_2 activity, total cholesterol, triglycerides, aspartyl aminotransferase (ASAT) and C-reactive protein (CRP) during the four week follow-up period after the gene transfer were measured.

Lp-PLA_2 activity assay kit was from Cayman Chemical and Lp-PLA_2 inhibitor Pefabloc (4-[2-aminoethyl]-

benzosulfonylfluoride) was purchased from Roche Diagnostics. Blood plasma values of total cholesterol, ASAT and CRP were measured on day 7 using routine clinical chemistry assays at the Kuopio University Hospital Laboratory.

Lipid peroxide content of LDL (150 μg) was estimated by measuring the TBARS produced in terms of malondialdehyde (MDA) (Ylä-Herttuala et al., 1989). Conjugated diene formation was measured from plasma samples as described previously (Turpeinen et al., 1995) and expressed as mmol/mol cholesterol.

4.6. RT-PCR

RT-PCR was used to detect expression of transferred gene in target tissues and also in peripheral tissues. Total RNA was isolated from the tissue samples after homogenisation in Trizol reagent (Gibco BRL) and treated with RQ1 RNAse-free DNAse (Promega). Four μg of total RNA was reverse-transcribed using random hexamer primers (Promega) and M-Mulv Reverse Transcriptase (New England BioLabs). cDNA was amplified by PCR using DyNAzyme™ II DNA Polymerase (Finnzymes). Details about PCR conditions and primers are presented in table 6.

construct	**primer sequences (forward, reverse)**	**annealing**	**cycles**
Lp-PLA_2	TGGAGCAACGGTTATTCAG, TGGTTGTGTTAATGTTGGTCC	62 ºC	45
35k	ATCCTCATCCTCCTCCTCGT, CTCAGACCTCCACCGATGAT	55 ºC	35
TIMP-1	ACCCAACGACGGCCTTCTGCAATTC, GGCTATCTGGGACCGCAGGGACTGC	55 ºC	35
LacZ	1) TGAGGGGACGACGACAGTAT, TTGGAGGCCTAGGCTTTTGC 2) GGTAGAAGACCCCAAGGACTTT, CGCCATTCGCCATTCAG	58 ºC 58 ºC	40 40
EC-SOD	1) TGATGTTGGGCGACCG, GGATGTTGCAAGTG ACCAGGC 2) GTGAGCGCCTGCCAGATCTC, GGATGTTGCAAGTG ACCAGGC	60 ºC 60 ºC	30 30

Table 6. PCR conditions

4.7. Lp-PLA_2 activity analysis

Serum and LDL Lp-PLA_2 activity was determined by using a commercially available assay kit according to manufacturer's instructions (Cayman Chemical). The assay uses 2-thio-PAF, which serves as a substrate for Lp-PLA_2. Upon hydrolysis of the acetyl thioester bond

by Lp-PLA_2, free thiols are detected using 5,5'-dithiobis-2-nitrobenzoic acid (Ellman's reagent). The absorbance is read at 414 nm over a period of time using an ELISA plate reader. Absorbance values were plotted as a function of time and the Lp-PLA_2 activity was calculated from the linear portion of the curve and expressed as $nmol \cdot ml^{-1} \cdot min^{-1}$.

4.8. Statistics

Chi-square test or ANOVA followed by modified t-test was used to evaluate statistical significances. A value of $P<0.05$ was considered statistically significant. Numerical values for each measurement are shown as mean±SEM.

5. Results

5.1. Cell culture studies

5.1.1. Transfection efficiency

Rabbit aortic SMCs (RAASMC) and RAW 264 cells were transduced with AdLp-PLA_2 in order to study the functionality of AdLp-PLA_2 construct in vitro. The maximal Lp-PLA_2 enzyme activity in the lyophilized medium showed over 10-fold increased activity in SMC and RAW 264 cells as compared to the activity in the untransduced controls.

5.1.2. Immunoblot analysis and RT-PCR

The total RNA was collected after 48 h for RT-PCR. The presence of Lp-PLA_2 transcript was detected in AdLp-PLA_2 transduced cells by using primers specific for human Lp-PLA_2. Identity of the correct PCR product was assessed by size fractionation on ethidium bromide-stained agarose gels.

Western blot analysis from lyophilised medium of the same cells showed in the AdLp-PLA_2 transduced cell supernatants but not in the untransduced control cell supernatants the presence of approximately 65 kDa protein, which corresponds to the molecular weight identified for the glycosylated form of serum Lp-PLA_2.

5.1.3. LDL oxidation

Effect of the increased Lp-PLA_2 activity on LDL oxidation and subsequent degradation in macrophages was analyzed using LDLs isolated from rabbits given different doses of AdLp-PLA_2. Agarose gel electrophoresis showed a slight, but not significant, decrease in the migration of ^{125}I-LDL isolated from AdLp-PLA_2 group (10^{10} pfu) as compared to the migration of control LacZ ^{125}I-LDL and

^{125}I-LDL treated with Lp-PLA$_2$ inhibitor Pefabloc.

5.1.4. LDL degradation and foam cell formation

Degradation in RAW 264 macrophages of the isolated LDL fractions subjected to standardized oxidation was followed at different time points (3, 6, 12 and 24 h). Increased Lp-PLA$_2$ content in LDL particles decreased the degradation of LDL after oxidation to 60-87% of the control LacZ LDL degradation. The values at different time points were 87% for 3h, 60% for 6h, 77% for 12h and 74% for 24h (data not show). Next we studied the degradation of LDL isolated from rabbits given different adenovirus doses. The cells were incubated with medium containing 10 μg/ml of LDL for 6 h. As a result, the increased Lp-PLA$_2$ activity in the rabbit LDLs decreased the degradation of LDL after oxidation to 63-87% of the LacZ control LDL values and the decrease was proportional to the virus dose and Lp-PLA$_2$ activity. Inhibition of the Lp-PLA$_2$ activity by Pefabloc led to a 2-fold increase in the degradation as compared to the LacZ control LDL. The inhibition of the degradation was most effective with LDL containing the highest level of Lp-PLA$_2$ activity.

To test whether the Lp-PLA$_2$ could inhibit the uptake of larger quantities of oxLDL, we tested the effect of increased Lp-PLA$_2$ activity on foam-cell formation in RAW 264 macrophages incubating the cells for 18 h with 100 μg/ml of LDLs isolated from the transduced rabbits and subjected to standardized oxidation. The lipid accumulation in RAW 264 macrophages and foam-cell formation was decreased when incubated with oxLDL containing the highest Lp-PLA$_2$ activity. When Lp-PLA$_2$ was irreversibly inhibited in the LDL particle, it led to an increase in the lipid accumulation and foam-cell formation as compared to the Lp-PLA$_2$ and LacZ control LDL groups.

5.2. Lp-PLA$_2$ activity in LDL particle

The gene transfer also led to increased Lp-PLA$_2$ activity in serum: at adenovirus dose 10^8 pfu (n=4) the activities were 49±1.4 nmol/min/ml in Lp-PLA$_2$ group and 42±2.8 nmol/min/ml in LacZ group; at dose 10^9 pfu (n=4) 51±4.9 nmol/min/ml in Lp-PLA$_2$ group and 38±4.2 nmol/min/ml in LacZ group and at dose 10^{10} pfu (n=4) 63±1.4 nmol/min/ml in Lp-PLA$_2$ group and 38±2.8 nmol/min/ml in LacZ group, respectively. Lp-PLA$_2$ activity was also measured from isolated rabbit LDL. LDL particles with 3-fold increased Lp-PLA$_2$ activity were produced with the highest dose of AdLp-PLA$_2$. An aliquot of the LDL from each dose group was treated with Pefabloc and Lp-PLA$_2$ inhibitor treatment irreversibly inhibited the Lp-PLA$_2$ activity in rabbit LDL.

5.3. Restenosis studies

5.3.1. Lp-PLA$_2$ and LacZ gene transfer

Plasma Lp-PLA$_2$ activity was measured before (day 0) and 3, 7, 14 and 28 days after AdLp-PLA$_2$ or AdLacZ gene transfer. Plasma Lp-PLA$_2$ activity showed a statistically significant increase in the Lp-PLA$_2$ group (48.2±4.2) as compared to the LacZ group (33.6±3.51, $p<0.05$) at two weeks time point. Lp-PLA$_2$ mRNA expression in aortas was detected two and four weeks after the gene transfer. Adenovirus biodistribution was determined from liver, spleen, kidney, lung and aorta. As expected, some positive x-gal staining was seen in spleen, liver, lung and aorta two weeks after the gene transfer.

5.3.2. Histological analysis

To determine the effect of gene transfers on neointima formation I/M ratio was measured from aortic samples. Histological analysis showed significantly ($p<0.005$) reduced neointimal thickening in the Lp-PLA$_2$ group as compared to the LacZ controls at two weeks time point. At four weeks time point I/M tended to be lower in the Lp-PLA$_2$ group as compared to the LacZ group ($p=0.057$). Results are seen in table 7 and in figure 4. Also unpublished data is included.

gene	**intima/media ratio**	
	2 wk	**4 wk**
LacZ	0.45±0.05 0,56±0,02	0.53±0.06 0,82±0,18
Lp-PLA$_2$	0.25±0.03 *	0.34±0.05
TIMP-1	0,37±0,07	0,39±0,03 *
VEGF-C	0,39±0,02	0,47±0,17
VEGF-A	0,44±0,12	0,73±0,26
TIMP-1+VEGF-C	0,38±0,08	0,43±0,01 *
VEGF-A+VEGF-C	0,20±0,07 *	0,86±0,14

Table 7. Intima/media ratios in restenosis studies. *P<0.05.

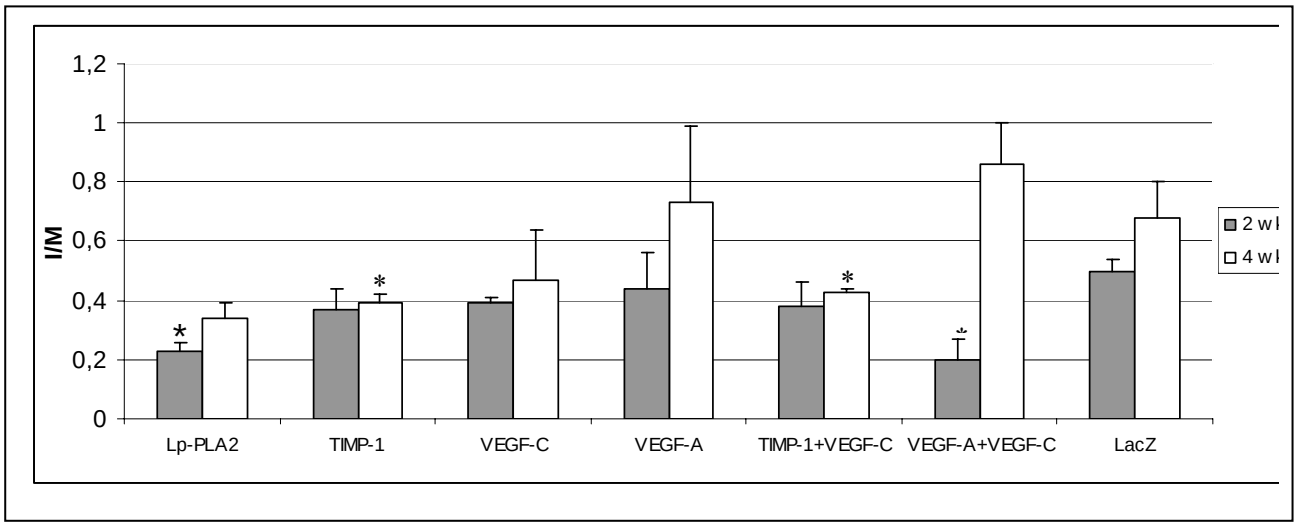

Figure 4. Intima/Media ratios in restenosis studies. * P<0.05.

RAM-11 staining showed no significant differences in the macrophage count between the groups (data not shown). Endothelial regrowth was analyzed by measuring the length of intact endothelium from histological sections. No significant differences were found between the groups in the regrowth of endothelium. Results are seen in table 8.

The percentage of proliferating cells was analyzed by BrdU labelling. No statistical differences were detected at two weeks time point, but the Lp-PLA_2 group tended to have a lower proliferation rate than the LacZ group. At four weeks time point the difference between the Lp-PLA_2 group and the LacZ group was significant ($p<0.05$). Results are seen in table 9.

Apoptosis was higher in the LacZ control vessels than in the Lp-PLA_2 group at two weeks time point, but the difference was not present at four weeks time point (data not shown).

gene	**regrowth of endothelium (%)**	
	2 wk	4 wk
LacZ	48.7±13.2	73.1±8.7
Lp-PLA_2	62.2±9.2	70.0±8.4

Table 8. Regrowth of endothelium in restenosis studies.

gene	labelling index (%)	
	2 wk	4 wk
LacZ	2.2±0.6	1.8±0.4
Lp-PLA$_2$	1.8±0.3	0.9±0.2 *

Table 9. The proliferation index in restenosis study. * P< 0.05.

5.3.2. Combination of adenoviral vectors

Study II showed that Lp-PLA$_2$ gene transfer reduced neointima formation in a rabbit restenosis model. When taking account the unpublished data showed earlier (figure 4), our results implicate that Lp-PLA$_2$ and TIMP-1 gene transfer alone is sufficient for decreasing neointima formation. When considering the gene combination used in figure 4, it is concluded that gene therapy with adenoviral Lp-PLA$_2$ or TIMP-1 alone is sufficient in reducing restenosis and that combination gene therapy does not bring any significant advantages.

5.4. Vein graft studies

5.4.1. LacZ gene transfer

The X-gal stainings showed transduction in the endothelium and intimal cells. In the biodistribution studies at two week time point positive lacZ staining was found only in the spleen. Thus, although gene transfer was made ex vivo and no adenovirus was exposed to systemic circulation. The biodistribution studies showed positive X-gal signals from spleen. This could be due to the local adenoviral exposure of the adventitial vessels. Stainings from lung and kidney did not show any signs of transduction.

5.4.2. Histological analysis

To determine the effects of gene transfers on neointima formation intima-media area ratios (I/M) were measured from vein graft samples. Results are seen in table 10 and in figure 5. These data suggest that combination therapy is needed for long-term results for decreased neointima formation.

gene	intima/media ratio	
	2 wk	**4 wk**
LacZ	0.42±0.05	0.37±0.07
35k	0.24±0.04 *	0.30±0.05
TIMP-1	0.30±0.1	0.32±0.04
EC-SOD	0.28±0.08	0.20±0.15
EC-SOD + 35k	0.19±0.10 *	0.35±0.21
EC-SOD + TIMP-1	0.18±0.02 *	0.16±0.03 *
TIMP-1 + 35k	0.27±0.12	0.23±0.02

Table 10. Intima/Media ratios in vein graft studies. * P<0.05.

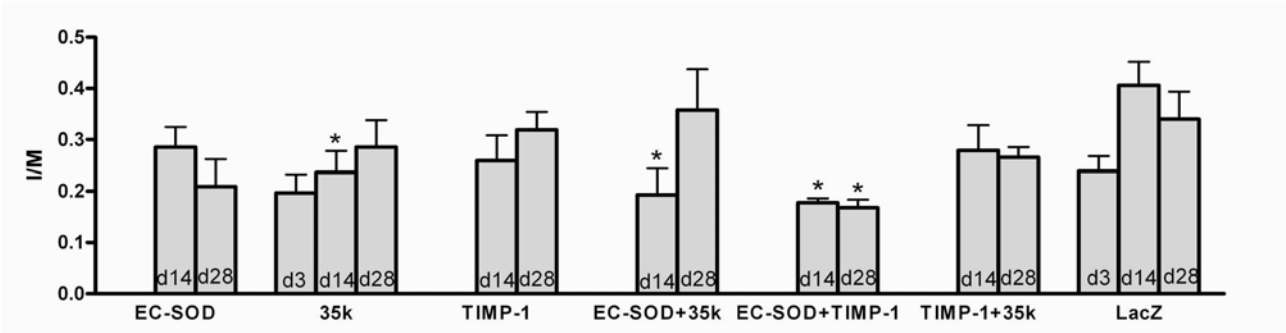

Figure 5. I/M ratios in vein graft studies. *P<0.05.

Macrophage accumulation is part of the inflammatory response in vein grafts. At three day time point no macrophages were present, probably due to the short period of time after the surgery. As expected, the anti- inflammatory combination group had the lowest macrophage account at both time points. Combined data is seen in table 11 and in figure 6.

gene	macrophage count	
	2 wk	**4 wk**
LacZ	96±61	174±123
35k	40±34 *	63±54
TIMP-1	117±112	195±177
EC-SOD	65±71	15±17
EC-SOD + 35k	14±25 **	12±14 *
EC-SOD + TIMP-1	123±178	62±81
TIMP-1 + 35k	59±78	62±57

Table 11. Results of macrophage counts in vein graft studies. *P<0.05; **P<0.01.

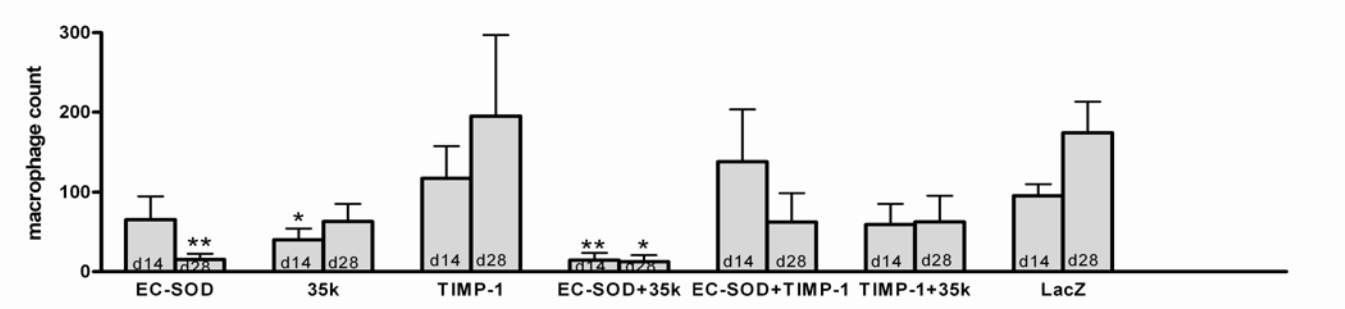

Figure 6. Results of macrophage counts in vein graft studies. *P<0.05; **P<0.01.

Surgical manipulation and rapid changes in blood pressure always cause some damages to the endothelium. Endothelial coverage in vein grafts after the gene transfer was analyzed by measuring the length of the intact endothelium from histological sections. At two weeks time point the AdEC-SOD (95.4 % ±5.0) group showed significantly ($p<0.05$) higher endothelial coverage as compared to the control group AdlacZ. At four weeks time point no statistically significant differences were present and the endothelial coverage tended to be at the same level in every treatment group. Data is seen in figure 7. Proliferating cells was measured by BrdU labelling (cells/mm^2). Data are seen in figure 8. It clearly shows that combination gene therapy lowers the proliferation in the vein grafts.

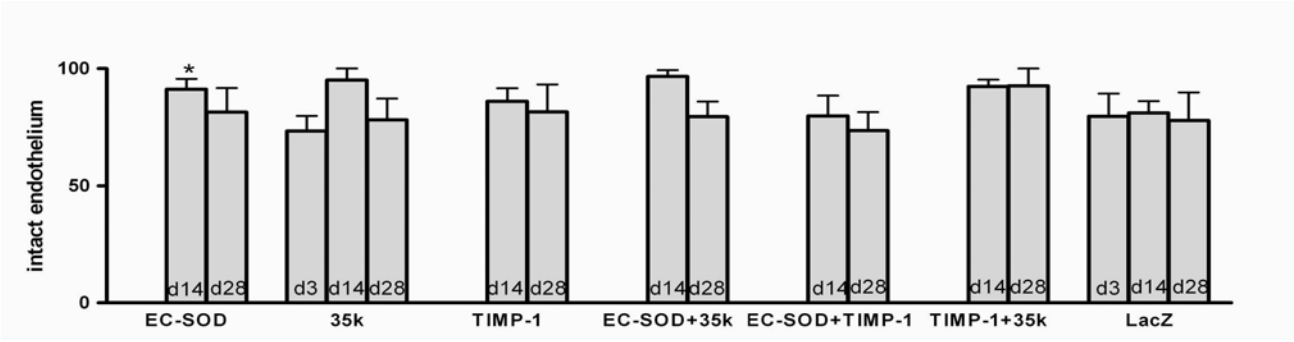

Figure 7. Endothelial coverage in vein grafts after the gene transfer. *P<0.05.

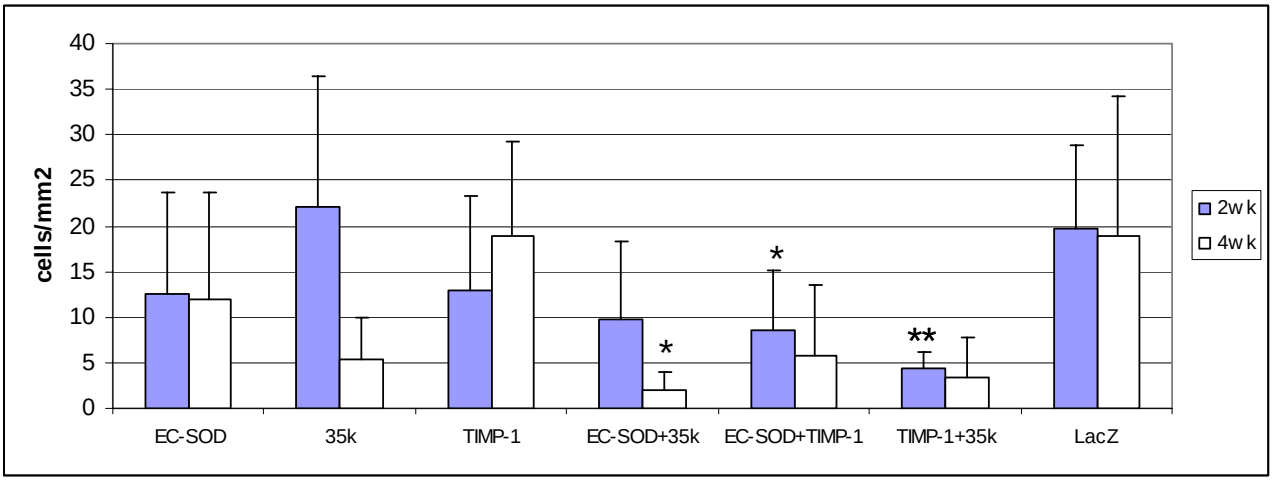

Figure 8. Measurement of the proliferation in vein grafts by BrdU staining. *P< 0.05; **P<0.01.

Apoptosis was detected by positive immunohistochemical scores (classes 1-2: low, 3-4: high staining). Apoptosis was degreased significantly in AdEC-SOD group in two and four weeks time points compared to LacZ. In addition, EC-SOD+35k gene transfer showed a significant reduction in apoptosis. At four week time point LacZ group showed increased apoptosis compared to all study groups. Results are seen in table 12.

a) **2 wk** positive staining	EC-SOD	35k	TIMP-1	EC-SOD+35k	EC-SOD+TIMP-1	TIMP-1+35k	LacZ
1-2 (low)	9	4	4	10	7	6	9
3-4 (high)	1	3	10	1	3	2	10
chi-test p	0.0245*			0.0171*			
b) **4 wk** positive staining	EC-SOD	35k	TIMP-1	EC-SOD+35k	EC-SOD+TIMP-1	TIMP-1+35k	LacZ
1-2 (low)	6	7	9	10	7	10	1
3-4 (high)	1	2	4	1	2	1	7
chi-test p	0.005**	0.007**	0.01*	0.0006**	0.007**	0.0006**	

Table 12. Apoptosis in vein graft studies.

6. Discussion

6.1. Role of Lp-PLA$_2$ in atherogenesis and restenosis

As mentioned earlier, the biological role of Lp-PLA$_2$ is to hydrolyse PAF and other polar phospholipids with a short, oxidized acyl chain in the *sn*-2 position of glycerol. It has been demonstrated that PAF-like lipids are generated during LDL oxidation and these lipids mimic PAF and can activate many types of cells via the PAF receptor and cause stimulation of platelet aggregation, leukocyte activation and adhesion to endothelium, increased vascular permeability, monocyte activation and production of superoxide anion by macrophages (Smiley et al., 1991). Lp-PLA$_2$ gene transfer could inhibit these events by hydrolyzing PAF-like lipids. The specificity of the enzyme for short, oxidized acyl groups ensures that phospholipid components of cellular membranes and lipoproteins remain intact, while products of oxidation and fragmentation are hydrolysed.

Lp-PLA$_2$ is a hydrophobic protein and in plasma 2/3 of the enzyme activity is associated with LDL, the rest of the activity being associated mainly with HDL (Stafforini et al., 1997). On the other hand, less than 1 % of the LDL particles contain Lp-PLA$_2$. Thus, even a minor increase in Lp-PLA$_2$ content could have important effects on the properties of LDL. Since it is difficult to get hydrophobic proteins in LDL in a test tube, we wanted to direct overexpression of Lp-PLA$_2$ into the liver where lipoprotein particles are produced. This was achieved with adenovirus-mediated gene transfer since adenovirus given via a systemic route is known to lead to strong transgene expression in the liver.

The main goal of the study I was to achieve a short time, effective Lp-PLA$_2$ overexpression in the liver for the production of LDLs with increased Lp-PLA$_2$ activity for in vitro studies. We considered these in vitro studies essential before trying any anti-atherosclerotic gene transfer protocols in rabbits. Extensive oxidation of LDL creates particles that are metabolized in macrophages by scavenger receptors, which leads to the accumulation of intracellular cholesterol. In study I LDL degradation and foam cell formation were used as biological indicators of the effects of increased Lp-PLA$_2$ activity. However, it should be kept in mind that both of these methods measure late stages in the sequence of LDL oxidation and require extensive modification of both apoB100 and lipid components of the studied LDL fractions. Nevertheless, we consider these analyze important surrogate markers of LDL atherogenicity in vivo, since increased intracellular cholesterol accumulation is the

hallmark of early human atherosclerotic lesions.

Protective effects of Lp-PLA_2 on the atherogenic properties of LDL are most likely related to its ability to destroy oxidatively fragmented phospholipids, which are one class of ligands on oxLDL for macrophage scavenger receptor recognition. Reduction of these substances can also prevent subsequent formation of fully oxLDL and uptake by macrophages. It has been shown that Lp-PLA_2 can prevent oxidative modification of LDL and mice transfected with human Lp-PLA_2 showed enzyme activity in lipoproteins, which became more resistant to copper induced oxidation with enhanced Lp-PLA_2 levels. Moreover, HDL-associated human Lp-PLA_2 inhibited foam cell formation and enhanced cholesterol efflux in macrophages (Noto et al., 2003).

On the other hand, action of Lp-PLA_2 on oxidized phosphatidylcholine (PC) produces lyso-PC which has been demonstrated to be atherogenic because of its chemoattracting potency for monocytes, inhibition of arterial relaxation induced by endothelium-derived relaxing factor and stimulation of several adhesion molecule and growth factor gene expression in EC:s (Murohara et al., 1994; Kume et al., 1992; Kume and Gimbrone, Jr., 1994). While these atherogenic effects of lyso-PC have been well described, most of the results are derived from experiments using commercially available lyso-PC compounds. A carefully performed trial demonstrated that most of the inflammatory activities of lyso-PC are caused by the minute amount of PAF contained in the compound and are no longer manifest after the removal of the contaminated PAF (Marathe et al., 2001). Thus, Lp-PLA_2 can potentially affect LDL oxidation process both in the early and late phases by reducing bioactive PAF-like lipids in minimally modified LDL and causing lyso-PC accumulation in the lipoprotein particle. However, it is important to note that lyso-PC is water soluble and can diffuse out from LDL. In addition, some antiatherogenic actions of lyso-PC have also been reported, for example the promotion of cholesterol efflux from macrophage foam cells (Hara et al., 1997). Therefore, it is possible that while protecting LDL particle from becoming oxidized, Lp-PLA_2 activity may lead to enhanced production of lyso-PC, which has multiple effects on atherogenesis.

When interpreting the results of the study I, it should be remembered that we have expressed human Lp-PLA_2 in rabbits and that binding of the human enzyme to rabbit LDL may not fully resemble human situation. Also, while preparing radiolabeled oxLDL for degradation studies lipoprotein preparations have been dialyzed and this could have reduced lyso-PC content in the lipoprotein particles modifying their biological properties. Lastly, inhibitor used for the study is not absolutely specific for Lp-PLA_2 and

may have additional unknown effects on LDL oxidation and lipid uptake by macrophages.

In the context of atherosclerosis, the role of Lp-PLA_2 is still somewhat unclear and is considered to have a dual role: one that is anti-inflammatory (Tjoelker and Stafforini, 2000) and one that is proinflammatory due to the generation of lyso-PC which is an abundant component of oxLDL (Quinn et al., 1988). Increased Lp-PLA_2 expression and activity has been demonstrated in human and rabbit atherosclerotic lesions (Häkkinen et al., 1999). However, it remains unclear whether Lp-PLA_2 contributes to the progression of human lesions and definitive conclusions about the pro- and antiatherogenic roles of Lp-PLA_2 activity and its inhibition can only be obtained from prospective human intervention studies.

Our results show that when subjected to in vitro oxidation, elevated levels of Lp-PLA_2 activity in LDL reduce subsequent lipoprotein degradation and foam cell formation in macrophages which suggest that in the early fatty streaks Lp-PLA_2 may have antiatherogenic effect by reducing proinflammatory changes and lipid uptake in lesion macrophages. In the study II we further investigated the effect of Lp-PLA_2 gene transfer in hypercholesterolemic restenosis rabbit model.

The protective effect of Lp-PLA_2 on restenosis is likely to be due to its ability to remove PAF and PAF-like oxidized phospholipids formed during and after arterial injury. It has been demonstrated that angioplasty is accompanied by platelet and leukocyte activation leading to increased PAF production and platelet-leukocyte adhesion (Mickelson et al., 1996; Neumann et al., 1996). PAF and PAF-like phospholipids are mitogens for SMCs and may thus contribute to the pathogenesis of restenosis (Stoll and Spector, 1989; Heery et al., 1995). The injury in the vessel wall was made by balloon-denudation and the gene transfer was directed to the site of balloon-injury. Balloon denudation immediately increases O_2^- production (Souza et al., 2000) and also hypercholesterolemia contributes to this (Ohara et al., 1993) suggesting a strong oxidant stress at the site of injury which can lead to formation of PAF-like lipids.

First evidence of an inhibitory effect of Lp-PLA_2 on intimal hyperplasia was shown by Quarck et al, who demonstrated that systematically administered adenovirus-mediated gene transfer of human Lp-PLA_2 inhibited injury-induced neointima formation and resulted in reduced adhesion molecule expression and monocyte adhesion in apolipoprotein E-deficient mice (Quarck et al., 2001). In our rabbit model we used intravascular catheter-mediated gene transfer method in order to achieve local production of the treatment gene in the vessel wall, but with secreted gene product also systemic effects were achieved as seen in this study with

elevated serum Lp-PLA_2 levels. Some Lp-PLA_2 adenovirus was also distributed into the liver and according to our previous studies this leads to the expression of Lp-PLA_2 in the liver and formation of LDL particles with increased Lp-PLA_2 activity which protects LDL against oxidative modification and degradation in macrophages (Turunen et al., 2004). A small portion of circulating enzyme activity is associated with HDL and there are several lines of evidence suggesting the antiatherogenic effects of Lp-PLA_2 bound to HDL (Quarck et al., 2001; Eisaf and Tselepis, 2003; Theilmeier et al., 2000).

Endothelial dysfunction can result in the release of growth factors promoting vascular cell proliferation. In study II Lp-PLA_2 gene transfer reduced apoptosis at two weeks time point. The exact role of apoptosis in restenosis in unknown, but at early stages after vascular injury apoptosis stimulates restenosis by provoking the wound-healing process. Apoptotic cells release cytokines and this could enhance the proliferative response after balloon injury (Walsh et al., 2000). There are many studies about the antiapoptotic effects of Lp-PLA_2 –like activity (Matsuzawa et al., 1997; Kuijpers et al., 2001) including the study by Chen et al, who showed that recombinant Lp-PLA_2, inhibited apoptosis induced by mildly oxidized LDL in cultured vascular endothelial cells and prevented calcium influx into neutrophils (Chen et al., 2003). This anti-apoptotic effect of Lp-PLA_2 could be important in the inhibition of neointima formation, because it has been shown that early inhibition of apoptosis after balloon injury reduces neointimal hyperplasia (Beohar et al., 2004). In addition to reducing apoptosis in study II, Lp-PLA_2 gene transfer lowered the proliferation rate which is in line with the observation that Lp-PLA_2 gene transfer led to the lower I/M ratios at both time points.

In conclusion, our results show that local catheter-mediated delivery of Lp-PLA_2 adenoviruses can reduce restenosis in rabbits and suggest that local administration of Lp-PLA_2 adenovirus could become a new approach for the prevention of restenosis after vascular manipulations.

6.2. Vein-graft gene therapy

Vein graft disease differs from arterial atherosclerosis in that its natural history is much shorter and the date of onset is clearly defined, i.e. graft implantation. This process is therefore potentially amenable to strategies that inhibit its progression. The ability to manipulate vein grafts ex vivo prior to implantation using pharmacological or other methods that may inhibit subsequent disease is a feature unique to vein graft disease. Vein graft disease is a promising target for gene therapy because veins can be transduced ex vivo before grafting (Ylä-Herttuala and Martin, 2000). Although a number of studies have addressed molecular and cellular events in arterial restenosis, very little is known about gene transfer to vein grafts. According to our knowledge, this is the first study comparing different genes in the same vector in the rabbit jugular vein graft model. Adenovirus mediated reporter gene experiments have been done in various animal models of vein graft disease (Channon et al., 1997), but according to our knowledge, this study is the first comparing several different therapeutic genes in a rabbit jugular vein grafts for longer period. We studied the effects of three different genes and compared their treatment efficacy in rabbit vein-graft model.

The selection of therapeutic genes was based on the pathophysiological mechanisms of stenosis. TIMP-1 has been previously shown to reduce neointima formation in arterial restenosis models and in a human saphenous vein organ culture model (George et al., 1998b; Dollery et al., 1999; Turunen et al., 2002). We assumed that TIMP-1 could also have an effect in the rabbit vein graft model. AdTIMP-1 group also served as a positive control and helped to evaluate the magnitude of the effect obtained by Ad35k which has 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 two weeks time point as compared to the control. However, at four weeks time point all treatment groups had reached the control group values in I/M ratio. Previous studies have shown that adenoviral transgene expression decreases after two weeks time point (Hiltunen et al., 2000) and therefore the result was not unexpected. Thus, only short term effects were achieved, which may be due to the transient nature of the adenoviral gene transfer effect.

In next study we used the combination gene therapy in order to reduce neointima formation more efficiently and to prolong the treatment effect in vein grafts. We also wanted to study the effects of anti-inflammatory EC-SOD, which has shown a

great promise decreasing restenosis in a rabbit model (Laukkanen et al., 2002). Using these gene combinations (TIMP-1+35k, EC-SOD+35k, TIMP-1+EC-SOD) we assumed, that long- term results could be achieved. To our knowledge there are no previous vein graft combination gene therapy studies. Results showed that the combination of EC-SOD+35k and EC-SOD+TIMP-1 resulted in more enhanced inhibition of neointima formation than TIMP-1 or 35k alone. In addition, the treatment effect was evident still at four week time point in EC-SOD+TIMP-1 group.

It was also found that macrophage accumulation was significantly decreased at four weeks time point in Ad35k group as compared to AdlacZ group. Also, Ad35k group showed a decreased SMC proliferation index at four weeks time point. This may be due to the anti-inflammatory properties of 35k. 35k can block the CC- cytokines which could reduce macrophage accumulation and SMC proliferation rate. At three day time point signs of SMC proliferation or macrophage accumulation were not yet detectable, this is probably due to the short time point after the operation. In a combination studies it was shown again that the combination had favorable effects. When combining two anti-inflammatory genes EC-SOD+35k it led to significant decrease in macrophage count still at four week time point comparing to single gene transfer results. Also, AdEC-SOD alone showed significant anti-inflammatory effects at four week time point suggesting its persistent anti-inflammatory effects. These findings indicate that oxidative stress may play an important role in the pathogenesis of vein graft stenosis

Decreased apoptosis was evident at four weeks time point both in 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 injury. 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 addition, there was effect on inhibiting proliferation using combination of treatment genes; combination of TIMP-1+35k and TIMP-1+EC-SOD showed decreased proliferation compared to single gene studies.

We found that combination gene therapy is effective in decreasing proliferation of neointima and anti-inflammatory mediators and the effect is seen still at four week time point. This may be because two different treatment genes can more efficiently affect pathological events at early stages and stop the degenerative domino effect more efficiently than just one gene transfer. In vein graft stenosis the pathological events happen early after operation and it is important to slow the degenerative caskade as soon as

possible and in many reaction points. When using proper gene combination cocktails, this can probably be achieved. In preventing vein graft stenosis and its pathological events these results clearly show that the combination of treatment genes is favorable.

Most graft stenosis occurs asymptomatically and early recognition would help to prevent further myocardial cell ischemia and damage. It is therefore important to identify the key mechanisms leading to graft failure in order to delay the disease process. New treatment methods need to be investigated. The use of phage display to identify a peptide that specifically homes to vein grafts may allow targeted gene therapy. Attachment of a gene or drug to this peptide would allow it to be given systemically. Also, the use of microarray technology could be applied to identify new candidate genes that are differentially expressed in vein graft atherosclerosis compared with normal vein tissue. Genetic preconditioning of the vein graft with gene transfer of an antioxidant molecule is attractive in theory but would be difficult in practice because of the immediate onset of reperfusion after implantation and the time delay necessary for adequate local expression of the protective gene product.

7. Summary and conclusions

1. Intravascular infusion of AdLp-PLA_2 led to the production of functional Lp-PLA_2 protein and the increase of enzyme activity in isolated LDL particles. The increase was dose-dependent to adenovirus titer. Lp-PLA_2 protein was detected in the liver by immunohistochemistry and in the plasma by activity assay. LDL isolated from the transduced animals and subjected to oxidation showed dose-dependent inhibition of degradation of oxLDL by murine macrophages. In addition, oxLDL from Lp-PLA_2 transduced animals was less likely to promote foam cell formation (I).

2. Intravascular adenovirus-mediated local gene transfer of Lp-PLA at the site of balloon injury resulted in reduced intimal thickening in rabbit aorta. In addition, gene transfer led to increased Lp-PLA_2 levels in the plasma and decreased proliferation at four week time point and apoptosis at two week time point (II).

3. In study III we established a rabbit vein graft model and studied effects of two different therapeutic genes on vein graft stenosis. We showed that adenovirus-mediated gene transfer of vaccinia-virus anti-inflammatory protein 35k reduces vein graft stenosis and TIMP-1 gene transfer was used as a positive control as it is known to have stenosis reducing effects. It was found that the anti-inflammatory protein 35k showed promising effects on reducing neointima formation, but at four week time point the study groups had the same intima/media levels as the control lacZ group. We conclude 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.

4. In study IV we used adenovirus-mediated combination gene therapy in order to reduce neointima formation more efficiently and to prolong the treatment effect in vein grafts. At two week time point the combination of Ad(EC-SOD+35k) and Ad(EC-SOD+TIMP-1) showed reduced levels of intimal hyperplasia compared to single gene studies. A long term effect was seen in Ad(EC-SOD+TIMP-1) group; the combination showed a persistent decrease in the stenosis rate suggesting that the combination of anti-inflammatory and anti-proliferative proteins may be beneficial in the treatment of vein graft stenosis. The combination of anti-inflammatory proteins Ad(EC-SOD+35k) was the most efficient in reducing macrophage accumulation which was still significant at four week time point. These findings indicate that oxidative stress may play an important role in the pathogenesis of vein graft stenosis. Also, combination therapy had favorable effects on inhibiting cell proliferation. Our study is the first one to compare multiple gene effects on the vein graft model in rabbit. We found out that combination gene therapy was more effective than single gene studies in decreasing proliferation and inflammatory effects in vein grafts.

8. References

Airenne,K.J., Hiltunen,M.O., Turunen,M.P., Turunen,A.M., Laitinen,O.H., Kulomaa,M.S., and Yla-Herttuala,S. (2000) Baculovirus-mediated periadventitial gene transfer to rabbit carotid artery. Gene Ther. *7*, 1499-1504.

Angelini,G.D., Bryan,A.J., Williams,H.M., Morgan,R., and Newby,A.C. (1990) Distention promotes platelet and leukocyte adhesion and reduces short-term patency in pig arteriovenous bypass grafts. J. Thorac. Cardiovasc. Surg. *99*, 433-439.

Angelini,G.D., Izzat,M.B., Bryan,A.J., and Newby,A.C. (1996) External stenting reduces early medial and neointimal thickening in a pig model of arteriovenous bypass grafting. J Thorac Cardiovasc Surg *112*, 79-84.

Angelini,G.D. and Jeremy,J.Y. (2002) Towards the treatment of saphenous vein bypass graft failure--a perspective of the Bristol Heart Institute. Biorheology *39*, 491-499.

Bellosta,S., Bernini,F., Ferri,N., Quarato,P., Canavesi,M., Arnaboldi,L., Fumagalli,R., Paoletti,R., and Corsini,A. (1998) Direct vascular effects of HMG-CoA reductase inhibitors. Atherosclerosis *137 Suppl*, S101-S109.

Beohar,N., Flaherty,J.D., Davidson,C.J., Maynard,R.C., Robbins,J.D., Shah,A.P., Choi,J.W., MacDonald,L.A., Jorgensen,J.P., Pinto,J.V., Chandra,S., Klaus,H.M., Wang,N.C., Harris,K.R., Decker,R., and Bonow,R.O. (2004) Antirestenotic effects of a locally delivered caspase inhibitor in a balloon injury model. Circulation *109*, 108-113.

Bilheimer,D.W., Eisenberg,S., and Levy,R.I. (1972) The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. Biochim. Biophys. Acta *260*, 212-221.

Bittl,J.A. (1996) Advances in coronary angioplasty [published erratum appears in N Engl J Med 1997 Feb 27;336(9):670]. N Engl J Med *335*, 1290-1302.

Boring,L., Gosling,J., Cleary,M., and Charo,I.F. (1998) Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. Nature *394*, 894-897.

Bourassa,M.G., Campeau,L., Lesperance,J., and Grondin,C.M. (1982) Changes in grafts and coronary arteries after saphenous vein aortocoronary bypass surgery: results at repeat angiography. Circulation *65*, 90-97.

Bursill,C.A., Cai,S., Channon,K.M., and Greaves,D.R. (2003) Adenoviral-mediated delivery of a viral chemokine binding protein blocks CC-chemokine activity in vitro and in vivo. Immunobiology *207*, 187-196.

Bursill,C.A., Choudhury,R.P., Ali,Z., Greaves,D.R., and Channon,K.M. (2004) Broad-spectrum CC-chemokine blockade by gene transfer inhibits macrophage recruitment and atherosclerotic plaque formation in apolipoprotein E-knockout mice. Circulation *110*, 2460-2466.

Campeau,L., Enjalbert,M., Lesperance,J., Bourassa,M.G., Kwiterovich,P., Jr., Wacholder,S., and Sniderman,A. (1984) The relation of risk factors to the development of atherosclerosis in saphenous-vein bypass grafts and the progression of disease in the native circulation. A study 10 years after aortocoronary bypass surgery. N. Engl. J. Med. *311*, 1329-1332.

Carew,T.E., Schwenke,D.C., and Steinberg,D. (1987) Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. Proc. Natl. Acad. Sci. U. S. A. *84*, 7725-7729.

Chang,M.W., Ohno,T., Gordon,D., Lu,M.M., Nabel,G.J., Nabel,E.G., and Leiden,J.M. (1995) Adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene inhibits vascular smooth muscle cell proliferation and neointima formation following balloon angioplasty of the rat carotid artery. Mol. Med. *1*, 172-181.

Channon,K.M., Fulton,G.J., Gray,J.L., Annex,B.H., Shetty,G.A., Blazing,M.A., Peters,K.G., Hagen,P.O., and George,S.E. (1997) Efficient adenoviral gene transfer to early venous bypass grafts: comparison with native vessels. Cardiovasc Res *35*, 505-513.

Chen,C.H., Jiang,T., Yang,J.H., Jiang,W., Lu,J., Marathe,G.K., Pownall,H.J., Ballantyne,C.M., McIntyre,T.M., Henry,P.D., and Yang,C.Y. (2003) Low-density lipoprotein in hypercholesterolemic human plasma induces vascular endothelial cell apoptosis by inhibiting fibroblast growth factor 2 transcription. Circulation *107*, 2102-2108.

Chen,S.J., Wilson,J.M., and Muller,D.W. (1994) Adenovirus-mediated gene transfer of soluble vascular cell adhesion molecule to porcine interposition vein grafts. Circulation *89*, 1922-1928.

Chikada,M. and Jones,M. (1999) Study of gene delivery in a rabbit vein graft model. Improvement of the efficiency of gene transfer into vein grafts. Jpn. J. Thorac. Cardiovasc. Surg. *47*, 204-209.

Cho,Y.W., Kim,J.D., and Park,K. (2003) Polycation gene delivery systems: escape from endosomes to cytosol. J. Pharm. Pharmacol. *55*, 721-734.

Christenson,J.T. (2001) Preoperative lipid control with simvastatin reduces the risk for graft failure already 1 year after myocardial revascularization. Cardiovasc. Surg. *9*, 33-43.

Clowes,A.W. (1986) The role of aspirin in enhancing arterial graft patency. J. Vasc. Surg. *3*, 381-385.

Clowes,A.W., Reidy,M.A., and Clowes,M.M. (1983) Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. Lab Invest *49*, 327-333.

Conte,M.S., Mann,M.J., Simosa,H.F., Rhynhart,K.K., and Mulligan,R.C. (2002) Genetic interventions for vein bypass graft disease: a review. J. Vasc. Surg. *36*, 1040-1052.

Cushing,G.L., Gaubatz,J.W., Nava,M.L., Burdick,B.J., Bocan,T.M., Guyton,J.R., Weilbaecher,D., DeBakey,M.E., Lawrie,G.M., and Morrisett,J.D. (1989) Quantitation and localization of apolipoproteins [a] and B in coronary artery bypass vein grafts resected at re-operation. Arteriosclerosis *9*, 593-603.

Cushing,S.D., Berliner,J.A., Valente,A.J., Territo,M.C., Navab,M., Parhami,F., Gerrity,R., Schwartz,C.J., and Fogelman,A.M. (1990) Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. Proc. Natl. Acad. Sci. U. S. A *87*, 5134-5138.

Dangas,G. and Fuster,V. (1996) Management of restenosis after coronary intervention. Am Heart J *132*, 428-436.

Davies,M.G. and Hagen,P.O. (1995) Pathophysiology of vein graft failure: a review. Eur. J. Vasc. Endovasc. Surg. *9*, 7-18.

Dentan,C., Tselepis,A.D., Chapman,M.J., and Ninio,E. (1996) Pefabloc, 4-[2-aminoethyl]benzenesulfonyl fluoride, is a new, potent nontoxic and irreversible inhibitor of PAF-degrading acetylhydrolase. Biochim. Biophys. Acta *1299*, 353-357.

Dollery,C.M., Humphries,S.E., McClelland,A., Latchman,D.S., and McEwan,J.R. (1999) Expression of tissue inhibitor of matrix metalloproteinases 1 by use of an adenoviral vector inhibits smooth muscle cell migration and reduces neointimal hyperplasia in the rat model of vascular balloon injury. Circulation *99*, 3199-3205.

Donners,M.M., Daemen,M.J., Cleutjens,K.B., and Heeneman,S. (2003) Inflammation and restenosis: implications for therapy. Ann. Med. *35*, 523-531.

Edelman,E.R. and Rogers,C. (1998) Pathobiologic responses to stenting. Am. J. Cardiol. *81*, 4E-6E.

Eisaf,M. and Tselepis,A.D. (2003) Effect of hypolipidemic drugs on lipoprotein-associated platelet activating factor acetylhydrolase. Implication for atherosclerosis. Biochem. Pharmacol. *66*, 2069-2073.

Eslami,M.H., Gangadharan,S.P., Belkin,M., Donaldson,M.C., Whittemore,A.D., and Conte,M.S. (2001) Monocyte adhesion to human vein grafts: a marker for occult intraoperative injury? J. Vasc. Surg. *34*, 923-929.

Farb,A., Weber,D.K., Kolodgie,F.D., Burke,A.P., and Virmani,R. (2002) Morphological predictors of restenosis after coronary stenting in humans. Circulation *105*, 2974-2980.

Fattori,R. and Piva,T. (2003) Drug-eluting stents in vascular intervention. Lancet *361*, 247-249.

Feliste,R., Perret,B., Braquet,P., and Chap,H. (1989) Protective effect of BN 52021, a specific antagonist of platelet-activating factor (PAF-acether) against diet-induced cholesteryl ester deposition in rabbit aorta. Atherosclerosis *78*, 151-158.

Finkel,T. (2003) Oxidant signals and oxidative stress. Curr. Opin. Cell Biol. *15*, 247-254.

Fischman,D.L., Leon,M.B., Baim,D.S., Schatz,R.A., Savage,M.P., Penn,I., Detre,K., Veltri,L., Ricci,D., and Nobuyoshi,M. (1994) A randomized comparison of coronary-stent placement and balloon angioplasty in the treatment of coronary artery disease. Stent Restenosis Study Investigators [see comments]. N. Engl. J. Med. *331*, 496-501.

Fitzgibbon,G.M., Kafka,H.P., Leach,A.J., Keon,W.J., Hooper,G.D., and Burton,J.R. (1996) Coronary bypass graft fate and patient outcome: angiographic follow-up of 5,065 grafts related to survival and reoperation in 1,388 patients during 25 years. J. Am. Coll. Cardiol. *28*, 616-626.

Flotte,T.R. and Carter,B.J. (1995) Adeno-associated virus vectors for gene therapy. Gene Ther. *2*, 357-362.

French,M.H. and Faxon,D.P. (2002) Update on radiation for restenosis. Rev. Cardiovasc. Med. *3*, 1-6.

Galis,Z.S. and Khatri,J.J. (2002) Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circ. Res. *90*, 251-262.

Gellman,J., Ezekowitz,M.D., Sarembock,I.J., Azrin,M.A., Nochomowitz,L.E., Lerner,E., and Haudenschild,C.C. (1991) Effect of lovastatin on intimal hyperplasia after balloon angioplasty: a study in an atherosclerotic hypercholesterolemic rabbit. J. Am. Coll. Cardiol. *17*, 251-259.

George,S.J. (1998) Tissue inhibitors of metalloproteinases and metalloproteinases in atherosclerosis. Curr Opin Lipidol *9*, 413-423.

George,S.J., Baker,A.H., Angelini,G.D., and Newby,A.C. (1998a) Gene transfer of tissue inhibitor of metalloproteinase-2 inhibits metalloproteinase activity and neointima formation in human saphenous veins. Gene Ther *5*, 1552-1560.

George,S.J., Johnson,J.L., Angelini,G.D., Newby,A.C., and Baker,A.H. (1998b) Adenovirus-mediated gene transfer of the human TIMP-1 gene inhibits smooth muscle cell migration and neointimal formation in human saphenous vein. Hum Gene Ther *9*, 867-877.

George,S.J., Lloyd,C.T., Angelini,G.D., Newby,A.C., and Baker,A.H. (2000) Inhibition of late vein graft neointima formation in human and porcine models by adenovirus-mediated overexpression of tissue inhibitor of metalloproteinase-3. Circulation *101*, 296-304.

George,S.J., Zaltsman,A.B., and Newby,A.C. (1997) Surgigal preparative injury and neointima formation increase MMP-9 expression and MMP-2 activation in human saphenous vein. Cardiovasc Res *33*, 447-459.

Gerszten,R.E., Mach,F., Sauty,A., Rosenzweig,A., and Luster,A.D. (2000) Chemokines, leukocytes, and atherosclerosis. J. Lab. Clin. Med. *136*, 87-92.

Glass,C.K. and Witztum,J.L. (2001) Atherosclerosis. the road ahead. Cell *104*, 503-516.

Godbey,W.T. and Mikos,A.G. (2001) Recent progress in gene delivery using non-viral transfer complexes. J. Control Release *72*, 115-125.

Goetzl,E.J., Banda,M.J., and Leppert,D. (1996) Matrix metalloproteinases in immunity. J. Immunol. *156*, 1-4.

Grines,C.L., Watkins,M.W., Helmer,G., Penny,W., Brinker,J., Marmur,J.D., West,A., Rade,J.J., Marrott,P., Hammond,H.K., and Engler,R.L. (2002) Angiogenic Gene Therapy (AGENT) trial in patients with stable angina pectoris. Circulation *105*, 1291-1297.

Gröner,A. (1986) **Specificity and safety of baculoviruses**. In The biology of baculoviruses, Vol1, R.R.Granados and B.A.Federici, eds. (Boca Raton: CRC Press), pp. 177-202.

Grube,E., Silber,S., Hauptmann,K.E., Mueller,R., Buellesfeld,L., Gerckens,U., and Russell,M.E. (2003) TAXUS I: six- and twelve-month results from a randomized, double-blind trial on a slow-release paclitaxel-eluting stent for de novo coronary lesions. Circulation *107*, 38-42.

Häkkinen,T., Luoma,J.S., Hiltunen,M.O., Macphee,C.H., Milliner,K.J., Patel,L., Rice,S.Q., Tew,D.G., Karkola,K., and Ylä-Herttuala,S. (1999) Lipoprotein-associated phospholipase A(2), platelet-activating factor acetylhydrolase, is expressed by macrophages in human and rabbit atherosclerotic lesions. Arterioscler. Thromb. Vasc. Biol *19*, 2909-2917.

Han,D.K., Haudenschild,C.C., Hong,M.K., Tinkle,B.T., Leon,M.B., and Liau,G. (1995) Evidence for apoptosis in human atherogenesis and in a rat vascular injury model. Am. J. Pathol. *147*, 267-277.

Hanke,H., Strohschneider,T., Oberhoff,M., Betz,E., and Karsch,K.R. (1990) Time course of smooth muscle cell proliferation in the intima and media of arteries following experimental angioplasty. Circ. Res. *67*, 651-659.

Hao,X., Mansson-Broberg,A., Gustafsson,T., Grinnemo,K.H., Blomberg,P., Siddiqui,A.J., Wardell,E., and Sylven,C. (2004) Angiogenic effects of dual gene transfer of bFGF and PDGF-BB after myocardial infarction. Biochem. Biophys. Res. Commun. *315*, 1058-1063.

Hara,S., Shike,T., Takasu,N., and Mizui,T. (1997) Lysophosphatidylcholine promotes cholesterol efflux from mouse macrophage foam cells. Arterioscler. Thromb. Vasc. Biol. *17*, 1258-1266.

Hedin,U. and Wahlberg,E. (1997) Gene therapy and vascular disease: potential applications in vascular surgery. Eur. J Vasc Endovasc. Surg. *13*, 101-111.

Hedman,M., Hartikainen,J., Syvanne,M., Stjernvall,J., Hedman,A., Kivela,A., Vanninen,E., Mussalo,H., Kauppila,E., Simula,S., Narvanen,O., Rantala,A., Peuhkurinen,K., Nieminen,M.S., Laakso,M., and Yla-Herttuala,S. (2003) Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT). Circulation *107*, 2677-2683.

Heery,J.M., Kozak,M., Stafforini,D.M., Jones,D.A., Zimmerman,G.A., McIntyre,T.M., and Prescott,S.M. (1995) Oxidatively modified LDL contains phospholipids with platelet-activating factor-like activity and stimulates the growth of smooth muscle cells. J. Clin. Invest. *96*, 2322-2330.

Henriksen,T., Mahoney,E.M., and Steinberg,D. (1983) Enhanced macrophage degradation of biologically modified low density lipoprotein. Arteriosclerosis *3*, 149-159.

Hermans,W.R., Rensing,B.J., Strauss,B.H., and Serruys,P.W. (1991) Prevention of restenosis after percutaneous transluminal coronary angioplasty: the search for a "magic bullet". Am. Heart J. *122*, 171-187.

Hevonoja,T., Pentikainen,M.O., Hyvonen,M.T., Kovanen,P.T., and la-Korpela,M. (2000) Structure of low density lipoprotein (LDL) particles: basis for understanding molecular changes in modified LDL. Biochim. Biophys. Acta *1488*, 189-210.

Hiltunen,M.O., Laitinen,M., Turunen,M.P., Jeltsch,M., Hartikainen,J., Rissanen,T.T., Laukkanen,J., Niemi,M., Kossila,M., Hakkinen,T.P., Kivela,A., Enholm,B., Mansukoski,H., Turunen,A.M., Alitalo,K., and Yla-Herttuala,S. (2000) Intravascular adenovirus-mediated VEGF-C gene transfer reduces neointima formation in balloon-denuded rabbit aorta. Circulation *102*, 2262-2268.

Hoare,J., Waddington,S., Thomas,H.C., Coutelle,C., and McGarvey,M.J. (2004) Complement inhibition rescued mice allowing observation of transgene expression following intraportal delivery of baculovirus in mice. J. Gene Med.

Horkko,S., Bird,D.A., Miller,E., Itabe,H., Leitinger,N., Subbanagounder,G., Berliner,J.A., Friedman,P., Dennis,E.A., Curtiss,L.K., Palinski,W., and Witztum,J.L. (1999) Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. J Clin Invest *103*, 117-128.

Huang,Y., Wang,L., Verweire,I., Qiang,B., Liu,X., Verbeken,E., Schacht,E., and De,S., I (2002) Optimization of local methylprednisolone delivery to inhibit inflammatory reaction and neointimal hyperplasia of coated coronary stents. J. Invasive. Cardiol. *14*, 505-513.

Imaizumi,T.A., Stafforini,D.M., Yamada,Y., McIntyre,T.M., Prescott,S.M., and Zimmerman,G.A. (1995) Platelet-activating factor: a mediator for clinicians. J. Intern. Med. *238*, 5-20.

Inoue,T., Sohma,R., Miyazaki,T., Iwasaki,Y., Yaguchi,I., and Morooka,S. (2000) Comparison of activation process of platelets and neutrophils after coronary stent implantation versus balloon angioplasty for stable angina pectoris. Am. J. Cardiol. *86*, 1057-1062.

Ip,J.H., Fuster,V., Israel,D., Badimon,L., Badimon,J., and Chesebro,J.H. (1991) The role of platelets, thrombin and hyperplasia in restenosis after coronary angioplasty. J. Am. Coll. Cardiol. *17*, 77B-88B.

Isner,J.M., Kearney,M., Bortman,S., and Passeri,J. (1995) Apoptosis in human atherosclerosis and restenosis. Circulation *91*, 2703-2711.

Isner,J.M., Walsh,K., Rosenfield,K., Schainfeld,R., Asahara,T., Hogan,K., and Pieczek,A. (1996) Arterial gene therapy for restenosis. Hum. Gene Ther. *7*, 989-1011.

Izzat,M.B., Mehta,D., Bryan,A.J., Reeves,B., Newby,A.C., and Angelini,G.D. (1996) Influence of external stent size on early medial and neointimal thickening in a pig model of saphenous vein bypass grafting. Circulation *94*, 1741-1745.

Janssens,S.P. (2003) Applied gene therapy in preclinical models of vascular injury. Curr. Atheroscler. Rep. *5*, 186-190.

Jones,K.A. and Peterlin,B.M. (1994) Control of RNA initiation and elongation at the HIV-1 promoter. Annu. Rev. Biochem. *63*, 717-743.

Kornowski,R., Hong,M.K., Tio,F.O., Bramwell,O., Wu,H., and Leon,M.B. (1998) In-stent restenosis: contributions of inflammatory responses and arterial injury to neointimal hyperplasia. J. Am. Coll. Cardiol. *31*, 224-230.

Kost,T.A. and Condreay,J.P. (1999) Recombinant baculoviruses as expression vectors for insect and mammalian cells. Curr. Opin. Biotechnol. *10*, 428-433.

Krieger,M., Acton,S., Ashkenas,J., Pearson,A., Penman,M., and Resnick,D. (1993) Molecular flypaper, host defense, and atherosclerosis. Structure, binding properties, and functions of macrophage scavenger receptors. J. Biol. Chem. *268*, 4569-4572.

Kuijpers,T.W., van den Berg,J.M., Tool,A.T., and Roos,D. (2001) The impact of platelet-activating factor (PAF)-like mediators on the functional activity of neutrophils: anti-inflammatory effects of human PAF-acetylhydrolase. Clin. Exp. Immunol. *123*, 412-420.

Kume,N., Cybulsky,M.I., and Gimbrone,M.A.J. (1992) Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. J Clin Invest *90*, 1138-1144.

Kume,N. and Gimbrone,M.A., Jr. (1994) Lysophosphatidylcholine transcriptionally induces growth factor gene expression in cultured human endothelial cells. J. Clin. Invest *93*, 907-911.

Kupfer,J.M., Ruan,X.M., Liu,G., Matloff,J., Forrester,J., and Chaux,A. (1994) High-efficiency gene transfer to autologous rabbit jugular vein grafts using adenovirus-transferrin/polylysine-DNA complexes. Hum. Gene Ther. *5*, 1437-1443.

Kutryk,M.J., Foley,D.P., van den,B.M., Hamburger,J.N., van der Giessen,W.J., Defeyter,P.J., Bruining,N., Sabate,M., and Serruys,P.W. (2002) Local intracoronary administration of antisense oligonucleotide against c-myc for the prevention of in-stent restenosis. Results of the randomized investigation by the thoraxcenter of antisense dna using local delivery and ivus after coronary stenting (ITALICS) trial. J. Am. Coll. Cardiol. *39*, 281-287.

Lafont,A., Guzman,L.A., Whitlow,P.L., Goormastic,M., Cornhill,J.F., and Chisolm,G.M. (1995) Restenosis after experimental angioplasty. Intimal, medial, and adventitial changes associated with constrictive remodeling. Circ. Res. *76*, 996-1002.

Laitinen,M., Hartikainen,J., Hiltunen,M.O., Eränen,J., Kiviniemi,M., Närvänen,O., Mäkinen,K., Manninen,H., Syvänne,M., Martin,J.F., Laakso,M., and Ylä-Herttuala,S. (2000) Catheter-mediated vascular endothelial growth factor gene transfer to human coronary arteries after angioplasty. Hum Gene Ther *11*, 263-270.

Laitinen,M., Mäkinen,K., Manninen,H., Matsi,P., Kossila,M., Agrawal,R.S., Pakkanen,T., Luoma,J.S., Viita,H., Hartikainen,J., Alhava,E., Laakso,M., and Ylä-Herttuala,S. (1998) Adenovirus-mediated gene transfer to lower limb artery of patients with chronic critical leg ischemia. Hum Gene Ther *9*, 1481-1486.

Landmesser,U., Merten,R., Spiekermann,S., Buttner,K., Drexler,H., and Hornig,B. (2000) Vascular extracellular superoxide dismutase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. Circulation *101*, 2264-2270.

Laukkanen,J., Lehtolainen,P., Gough,P.J., Greaves,D.R., Gordon,S., and Yla-Herttuala,S. (2000a) Adenovirus-mediated gene transfer of a secreted form of human macrophage scavenger receptor inhibits modified low-density lipoprotein degradation and foam-cell formation in macrophages. Circulation *101*, 1091-1096.

Laukkanen,M.O., Kivela,A., Rissanen,T., Rutanen,J., Karkkainen,M.K., Leppanen,O., Brasen,J.H., and Yla-Herttuala,S. (2002) Adenovirus-mediated extracellular superoxide dismutase gene therapy reduces neointima formation in balloon-denuded rabbit aorta. Circulation *106*, 1999-2003.

Laukkanen,M.O., Lehtolainen,P., Turunen,P., Aittomaki,S., Oikari,P., Marklund,S.L., and Yla-Herttuala,S. (2000b) Rabbit extracellular superoxide dismutase: expression and effect on LDL oxidation. Gene *254*, 173-179.

Le Breton,H., Plow,E.F., and Topol,E.J. (1996) Role of platelets in restenosis after percutaneous coronary revascularization. J. Am. Coll. Cardiol. *28*, 1643-1651.

Lee,Y.J., Daida,H., Yokoi,H., Miyano,H., Takaya,J., Sakurai,H., Mokuno,H., and Yamaguchi,H. (1996) Effectiveness of probucol in preventing restenosis after percutaneous transluminal coronary angioplasty. Jpn. Heart J. *37*, 327-332.

Lehr,H.A., Weyrich,A.S., Saetzler,R.K., Jurek,A., Arfors,K.E., Zimmerman,G.A., Prescott,S.M., and McIntyre,T.M. (1997) Vitamin C blocks inflammatory platelet-activating factor mimetics created by cigarette smoking. J. Clin. Invest *99*, 2358-2364.

Leon,M.B., Teirstein,P.S., Moses,J.W., Tripuraneni,P., Lansky,A.J., Jani,S., Wong,S.C., Fish,D., Ellis,S., Holmes,D.R., Kerieakes,D., and Kuntz,R.E. (2001) Localized intracoronary gamma-radiation therapy to inhibit the recurrence of restenosis after stenting. N. Engl. J. Med. *344*, 250-256.

Lever,A.M. (2000) Lentiviral vectors: progress and potential. Curr. Opin. Mol. Ther. *2*, 488-496.

Libby,P. (2000) Changing concepts of atherogenesis. J. Intern. Med. *247*, 349-358.

Libby,P. and Simon,D.I. (2001) Inflammation and thrombosis: the clot thickens. Circulation *103*, 1718-1720.

Libby,P., Sukhova,G., Lee,R.T., and Galis,Z.S. (1995) Cytokines regulate vascular functions related to stability of the atherosclerotic plaque. J. Cardiovasc. Pharmacol. *25 Suppl 2*, S9-12.

Lindner,V. and Reidy,M.A. (1991) Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. Proc. Natl. Acad. Sci. U. S. A *88*, 3739-3743.

Liu,M. and Subbaiah,P.V. (1994) Hydrolysis and transesterification of platelet-activating factor by lecithin-cholesterol acyltransferase. Proc. Natl. Acad. Sci. U. S. A *91*, 6035-6039.

Lowry,O.H., Rosenbrough,N.J., Farr,A.L., and Randall,R.J. (1951) Protein measurement with the Folin phenol reagent. J Biol Chem *193*, 265-275.

Lusis,A.J. (2000) Atherosclerosis. Nature *407*, 233-241.

Lynch,C.M., Hara,P.S., Leonard,J.C., Williams,J.K., Dean,R.H., and Geary,R.L. (1997) Adeno-associated virus vectors for vascular gene delivery. Circ. Res. *80*, 497-505.

Mann,M.J. and Conte,M.S. (2003) Transcription factor decoys for the prevention of vein bypass graft failure. Am. J. Cardiovasc. Drugs *3*, 79-85.

Mann,M.J., Whittemore,A.D., Donaldson,M.C., Belkin,M., Conte,M.S., Polak,J.F., Orav,E.J., Ehsan,A., Dell'Acqua,G., and Dzau,V.J. (1999) Ex-vivo gene therapy of human vascular bypass grafts with E2F decoy: the PREVENT single-centre, randomised, controlled trial. Lancet *354*, 1493-1498.

Marathe,G.K., Harrison,K.A., Murphy,R.C., Prescott,S.M., Zimmerman,G.A., and McIntyre,T.M. (2000) Bioactive phospholipid oxidation products. Free Radic. Biol. Med. *28*, 1762-1770.

Marathe,G.K., Silva,A.R., de Castro Faria Neto HC, Tjoelker,L.W., Prescott,S.M., Zimmerman,G.A., and McIntyre,T.M. (2001) Lysophosphatidylcholine and lyso-PAF display PAF-like activity derived from contaminating phospholipids. J. Lipid Res. *42*, 1430-1437.

Marmur,J.D., Poon,M., Rossikhina,M., and Taubman,M.B. (1992) Induction of PDGF-responsive genes in vascular smooth muscle. Implications for the early response to vessel injury. Circulation *86*, III53-III60.

Marx,S.O. and Marks,A.R. (2001) Bench to bedside: the development of rapamycin and its application to stent restenosis. Circulation *104*, 852-855.

Masaki,T., Rathi,R., Zentner,G., Leypoldt,J.K., Mohammad,S.F., Burns,G.L., Li,L., Zhuplatov,S., Chirananthavat,T., Kim,S.J., Kern,S., Holman,J., Kim,S.W., and Cheung,A.K. (2004) Inhibition of neointimal hyperplasia in vascular grafts by sustained perivascular delivery of paclitaxel. Kidney Int. *66*, 2061-2069.

Matsumoto,T., Komori,K., Yonemitsu,Y., Morishita,R., Sueishi,K., Kaneda,Y., and Sugimachi,K. (1998) Hemagglutinating virus of Japan-liposome-mediated gene transfer of endothelial cell nitric oxide synthase inhibits intimal hyperplasia of canine vein grafts under conditions of poor runoff. J. Vasc. Surg. *27*, 135-144.

Matsuzawa,A., Hattori,K., Aoki,J., Arai,H., and Inoue,K. (1997) Protection against oxidative stress-induced cell death by intracellular platelet-activating factor-acetylhydrolase II. J. Biol. Chem. *272*, 32315-32320.

Mehta,D., Angelini,G.D., and Bryan,A.J. (1996) Experimental models of accelerated atherosclerosis syndromes. Int. J. Cardiol. *56*, 235-257.

Mehta,D., George,S.J., Jeremy,J.Y., Izzat,M.B., Southgate,K.M., Bryan,A.J., Newby,A.C., and Angelini,G.D. (1998) External stenting reduces long-term medial and neointimal thickening and platelet derived growth factor expression in a pig model of arteriovenous bypass grafting. Nature Med *4*, 235-239.

Mickelson,J.K., Lakkis,N.M., Villarreal-Levy,G., Hughes,B.J., and Smith,C.W. (1996) Leukocyte activation with platelet adhesion after coronary angioplasty: a mechanism for recurrent disease? J. Am. Coll. Cardiol. *28*, 345-353.

Montrucchio,G., Alloatti,G., and Camussi,G. (2000) Role of platelet-activating factor in cardiovascular pathophysiology. Physiol Rev. *80*, 1669-1699.

Moreno,P.R., Falk,E., Palacios,I.F., Newell,J.B., Fuster,V., and Fallon,J.T. (1994) Macrophage infiltration in acute coronary syndromes. Implications for plaque rupture. Circulation *90*, 775-778.

Morishita,R., Gibbons,G.H., Horiuchi,M., Ellison,K.E., Nakama,M., Zhang,L., Kaneda,Y., Ogihara,T., and Dzau,V.J. (1995) A gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation in vivo. Proc. Natl. Acad. Sci. U. S. A *92*, 5855-5859.

Morishita,R., Higaki,J., Tomita,N., and Ogihara,T. (1998) Application of transcription factor "decoy" strategy as means of gene therapy and study of gene expression in cardiovascular disease. Circ. Res. *82*, 1023-1028.

Motwani,J.G. and Topol,E.J. (1998) Aortocoronary saphenous vein graft disease: Pathogenesis, predisposition, and prevention. Circulation *97*, 916-931.

Mueller,H.W., Haught,C.A., McNatt,J.M., Cui,K., Gaskell,S.J., Johnston,D.A., and Willerson,J.T. (1995) Measurement of platelet-activating factor in a canine model of coronary thrombosis and in endarterectomy samples from patients with advanced coronary artery disease. Circ. Res. *77*, 54-63.

Muller,D.W., Ellis,S.G., and Topol,E.J. (1992) Experimental models of coronary artery restenosis. J. Am. Coll. Cardiol. *19*, 418-432.

Murohara,T., Kugiyama,K., Ohgushi,M., Sugiyama,S., Ohta,Y., and Yasue,H. (1994) LPC in oxidized LDL elicits vasocontraction and inhibits endothelium- dependent relaxation. Am. J. Physiol. *267*, H2441-9.

Neumann,F.J., Ott,I., Gawaz,M., Puchner,G., and Schomig,A. (1996) Neutrophil and platelet activation at balloon-injured coronary artery plaque in patients undergoing angioplasty. J. Am. Coll. Cardiol. *27*, 819-824.

Noto,H., Hara,M., Karasawa,K., Iso,O., Satoh,H., Togo,M., Hashimoto,Y., Yamada,Y., Kosaka,T., Kawamura,M., Kimura,S., and Tsukamoto,K. (2003) Human plasma platelet-activating factor acetylhydrolase binds to all the murine lipoproteins, conferring protection against oxidative stress. Arterioscler. Thromb. Vasc. Biol. *23*, 829-835.

Ohara,Y., Peterson,T.E., and Harrison,D.G. (1993) Hypercholesterolemia increases endothelial superoxide anion production. J. Clin. Invest *91*, 2546-2551.

Pakkanen,T.M., Laitinen,M., Hippeläinen,M., Kallionpää,H., Lehtolainen,P., Leppänen,P., Luoma,J.S., Tarvainen,R., Alhava,E., and Ylä-Herttuala,S. (1999) Enhanced plasma cholesterol lowering effect of retrovirus- mediated LDL receptor gene transfer to WHHL rabbit liver after improved surgical technique and stimulation of hepatocyte proliferation by combined partial liver resection and thymidine kinase ganciclovir treatment. Gene Ther. *6*, 34-41.

Pasceri,V., Willerson,J.T., and Yeh,E.T. (2000) Direct proinflammatory effect of C-reactive protein on human endothelial cells. Circulation *102*, 2165-2168.

Perlman,H., Maillard,L., Krasinski,K., and Walsh,K. (1997) Evidence for the rapid onset of apoptosis in medial smooth muscle cells after balloon injury. Circulation *95*, 981-987.

Pietersma,A., Kofflard,M., de Wit,L.E., Stijnen,T., Koster,J.F., Serruys,P.W., and Sluiter,W. (1995) Late lumen loss after coronary angioplasty is associated with the activation status of circulating phagocytes before treatment. Circulation *91*, 1320-1325.

Pollard,V.W. and Malim,M.H. (1998) The HIV-1 Rev protein. Annu. Rev. Microbiol. *52*, 491-532.

Popma,J.J., Califf,R.M., and Topol,E.J. (1991) Clinical trials of restenosis after coronary angioplasty [editorial]. Circulation *84*, 1426-1436.

Popma,J.J., Suntharalingam,M., Lansky,A.J., Heuser,R.R., Speiser,B., Teirstein,P.S., Massullo,V., Bass,T., Henderson,R., Silber,S., von,R.P., Bonan,R., Ho,K.K., Osattin,A., and Kuntz,R.E. (2002) Randomized trial of 90Sr/90Y beta-radiation versus placebo control for treatment of in-stent restenosis. Circulation *106*, 1090-1096.

Porter,K.E. and Turner,N.A. (2002) Statins for the prevention of vein graft stenosis: a role for inhibition of matrix metalloproteinase-9. Biochem. Soc. Trans. *30*, 120-126.

Post,M.J., Borst,C., and Kuntz,R.E. (1994) The relative importance of arterial remodeling compared with intimal hyperplasia in lumen renarrowing after balloon angioplasty. A study in the normal rabbit and the hypercholesterolemic Yucatan micropig. Circulation *89*, 2816-2821.

Powell,J.S., Clozel,J.P., Muller,R.K., Kuhn,H., Hefti,F., Hosang,M., and Baumgartner,H.R. (1989) Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. Science *245*, 186-188.

Prescott,S.M., Zimmerman,G.A., Stafforini,D.M., and McIntyre,T.M. (2000) Platelet-activating factor and related lipid mediators. Annu. Rev. Biochem. *69*, 419-445.

Putzer,B.M., Bramson,J.L., Addison,C.L., Hitt,M., Siegel,P.M., Muller,W.J., and Graham,F.L. (1998) Combination therapy with interleukin-2 and wild-type p53 expressed by adenoviral vectors potentiates tumor regression in a murine model of breast cancer. Hum. Gene Ther. *9*, 707-718.

Puumalainen,A.M., Vapalahti,M., Agrawal,R.S., Kossila,M., Laukkanen,J., Lehtolainen,P., Viita,H., Paljärvi,L., Vanninen,R., and Ylä-Herttuala,S. (1998) beta-galactosidase gene transfer to human malignant glioma in vivo using replication-deficient retroviruses and adenoviruses. Hum Gene Ther *9*, 1769-1774.

Quarck,R., De Geest,B., Stengel,D., Mertens,A., Lox,M., Theilmeier,G., Michiels,C., Raes,M., Bult,H., Collen,D., Van Veldhoven,P., Ninio,E., and Holvoet,P. (2001) Adenovirus-mediated gene transfer of human platelet-activating factor-acetylhydrolase prevents injury-induced neointima formation and reduces spontaneous atherosclerosis in apolipoprotein E-deficient mice. Circulation *103*, 2495-2500.

Quinn,M.T., Parthasarathy,S., Fong,L.G., and Steinberg,D. (1987) Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis. Proc. Natl. Acad. Sci. U. S. A. *84*, 2995-2998.

Quinn,M.T., Parthasarathy,S., and Steinberg,D. (1988) Lysophosphatidylcholine: a chemotactic factor for human monocytes and its potential role in atherogenesis. Proc. Natl. Acad. Sci. U. S. A *85*, 2805-2809.

Quinonez,R. and Sutton,R.E. (2002) Lentiviral vectors for gene delivery into cells. DNA Cell Biol. *21*, 937-951.

Raines,E.W., Rosenfeld,M.E., and Ross,R. (1996) **The role of macrophages**. In Atherosclerosis and Coronary Artery Disease, V.Fuster, R.Ross, and E.J.Topol, eds. (Philadelphia: Lippincott-Raven Publishers), pp. 539-555.

Rajavashisth,T.B., Andalibi,A., Territo,M.C., Berliner,J.A., Navab,M., Fogelman,A.M., and Lusis,A.J. (1990) Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. Nature *344*, 254-257.

Rao,G.N. and Berk,B.C. (1992) Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression. Circ. Res. *70*, 593-599.

Ratliff,N.B. and Myles,J.L. (1989) Rapidly progressive atherosclerosis in aortocoronary saphenous vein grafts. Possible immune-mediated disease. Arch. Pathol. Lab Med. *113*, 772-776.

Rodrigo,L., Mackness,B., Durrington,P.N., Hernandez,A., and Mackness,M.I. (2001) Hydrolysis of platelet-activating factor by human serum paraoxonase. Biochem. J. *354*, 1-7.

Rome,J.J., Shayani,V., Flugelman,M.Y., Newman,K.D., Farb,A., Virmani,R., and Dichek,D.A. (1994) Anatomic barriers influence the distribution of in vivo gene transfer into the arterial wall. Modeling with microscopic tracer particles and verification with a recombinant adenoviral vector. Arterioscler. Thromb. *14*, 148-161.

Ross,R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature *362*, 801-809.

Ross,R. (1999) Mechanisms of disease - Atherosclerosis - An inflammatory disease. N Engl J Med *340*, 115-126.

Rutanen,J., Rissanen,T.T., Kivela,A., Vajanto,I., and Yla-Herttuala,S. (2001) Clinical Applications of Vascular Gene Therapy. Curr. Cardiol. Rep. *3*, 29-36.

Salonen,J.T., Salonen,R., Seppanen,K., Kantola,M., Suntioinen,S., and Korpela,H. (1991) Interactions of serum copper, selenium, and low density lipoprotein cholesterol in atherogenesis. BMJ *302*, 756-760.

Sarembock,I.J., Gertz,S.D., Gimple,L.W., Owen,R.M., Powers,E.R., and Roberts,W.C. (1991) Effectiveness of recombinant desulphatohirudin in reducing restenosis after balloon angioplasty of atherosclerotic femoral arteries in rabbits. Circulation *84*, 232-243.

Sarkis,C., Serguera,C., Petres,S., Buchet,D., Ridet,J.L., Edelman,L., and Mallet,J. (2000) Efficient transduction of neural cells in vitro and in vivo by a baculovirus-derived vector. Proc. Natl. Acad. Sci. U. S. A *97*, 14638-14643.

Schachner,T., Zou,Y., Oberhuber,A., Tzankov,A., Mairinger,T., Laufer,G., and Bonatti,J.O. (2004) Local application of rapamycin inhibits neointimal hyperplasia in experimental vein grafts. Ann. Thorac. Surg. *77*, 1580-1585.

Scheinman,M., Ascher,E., Levi,G.S., Hingorani,A., Shirazian,D., and Seth,P. (1999) p53 gene transfer to the injured rat carotid artery decreases neointimal formation. J Vasc Surg *29*, 360-369.

Schillinger,M., Exner,M., Mlekusch,W., Rumpold,H., Ahmadi,R., Sabeti,S., Haumer,M., Wagner,O., and Minar,E. (2002) Vascular inflammation and percutaneous transluminal angioplasty of the femoropopliteal artery: association with restenosis. Radiology *225*, 21-26.

Schwartz,R.S., Chronos,N.A., and Virmani,R. (2004) Preclinical restenosis models and drug-eluting stents: still important, still much to learn. J. Am. Coll. Cardiol. *44*, 1373-1385.

Schwartz,R.S., Huber,K.C., Murphy,J.G., Edwards,W.D., Camrud,A.R., Vlietstra,R.E., and Holmes,D.R. (1992) Restenosis and the proportional neointimal response to coronary artery injury: results in a porcine model. J. Am. Coll. Cardiol. *19*, 267-274.

Serrano,C.V., Jr., Ramires,J.A., Venturinelli,M., Arie,S., D'Amico,E., Zweier,J.L., Pileggi,F., and da Luz,P.L. (1997) Coronary angioplasty results in leukocyte and platelet activation with adhesion molecule expression. Evidence of inflammatory responses in coronary angioplasty. J. Am. Coll. Cardiol. *29*, 1276-1283.

Serruys,P.W., de Jaegere,P., Kiemeneij,F., Macaya,C., Rutsch,W., Heyndrickx,G., Emanuelsson,H., Marco,J., Legrand,V., and Materne,P. (1994) A comparison of balloon-expandable-stent implantation with balloon angioplasty in patients with coronary artery disease. Benestent Study Group. N. Engl. J. Med. *331*, 489-495.

Serruys,P.W., Herrman,J.P., Simon,R., Rutsch,W., Bode,C., Laarman,G.J., van,D.R., van den Bos,A.A., Umans,V.A., and Fox,K.A. (1995) A comparison of hirudin with heparin in the prevention of restenosis after coronary angioplasty. Helvetica Investigators. N. Engl. J. Med. *333*, 757-763.

Serruys,P.W., Luijten,H.E., Beatt,K.J., Geuskens,R., de Feyter,P.J., van den Brand,M., Reiber,J.H., ten Katen,H.J., van Es,G.A., and Hugenholtz,P.G. (1988) Incidence of restenosis after successful coronary angioplasty: a time- related phenomenon. A quantitative angiographic study in 342 consecutive patients at 1, 2, 3, and 4 months. Circulation *77*, 361-371.

Shears,L.L., Kibbe,M.R., Murdock,A.D., Billiar,T.R., Lizonova,A., Kovesdi,I., Watkins,S.C., and Tzeng,E. (1998) Efficient inhibition of intimal hyperplasia by adenovirus-mediated inducible nitric oxide synthase gene transfer to rats and pigs in vivo. J Am Coll. Surg *187*, 295-306.

Shi,Y., Pieniek,M., Fard,A., Obrien,J., Mannion,J.D., and Zalewski,A. (1996) Adventitial remodeling after coronary arterial injury. Circulation *93*, 340-348.

Shukla,N., Angelini,G.D., Ascione,R., Talpahewa,S., Capoun,R., and Jeremy,J.Y. (2003) Nitric oxide donating aspirins: novel drugs for the treatment of saphenous vein graft failure. Ann. Thorac. Surg. *75*, 1437-1442.

Siddiqui,A.J., Blomberg,P., Wardell,E., Hellgren,I., Eskandarpour,M., Islam,K.B., and Sylven,C. (2003) Combination of angiopoietin-1 and vascular endothelial growth factor gene therapy enhances arteriogenesis in the ischemic myocardium. Biochem. Biophys. Res. Commun. *310*, 1002-1009.

Simons,M., Edelman,E.R., DeKeyser,J.L., Langer,R., and Rosenberg,R.D. (1992) Antisense c-myb oligonucleotides inhibit intimal arterial smooth muscle cell accumulation in vivo. Nature *359*, 67-70.

Simonton,C.A., Leon,M.B., Baim,D.S., Hinohara,T., Kent,K.M., Bersin,R.M., Wilson,B.H., Mintz,G.S., Fitzgerald,P.J., Yock,P.G., Popma,J.J., Ho,K.K., Cutlip,D.E., Senerchia,C., and Kuntz,R.E. (1998) 'Optimal' directional coronary atherectomy: final results of the Optimal Atherectomy Restenosis Study (OARS). Circulation *97*, 332-339.

Smiley,P.L., Stremler,K.E., Prescott,S.M., Zimmerman,G.A., and McIntyre,T.M. (1991) Oxidatively fragmented phosphatidylcholines activate human neutrophils through the receptor for platelet-activating factor. J. Biol. Chem. *266*, 11104-11110.

Souza,H.P., Souza,L.C., Anastacio,V.M., Pereira,A.C., Junqueira,M.L., Krieger,J.E., da Luz,P.L., Augusto,O., and Laurindo,F.R. (2000) Vascular oxidant stress early after balloon injury: evidence for increased NAD(P)H oxidoreductase activity. Free Radic. Biol. Med. *28*, 1232-1242.

Springer,T.A. (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell *76*, 301-314.

Stafforini,D.M., McIntyre,T.M., Zimmerman,G.A., and Prescott,S.M. (1997) Platelet-activating factor acetylhydrolases. J. Biol. Chem. *272*, 17895-17898.

Stafforini,D.M., Zimmerman,G.A., McIntyre,T.M., and Prescott,S.M. (1992) The platelet-activating factor acetylhydrolase from human plasma prevents oxidative modification of low-density lipoprotein. Trans. Assoc. Am. Physicians *105*, 44-63.

Stary,H.C. (2000) Natural history of calcium deposits in atherosclerosis progression and regression. Z. Kardiol. *89 Suppl 2*, 28-35.

Steinbrecher,U.P. (1999) Receptors for oxidized low density lipoprotein. Biochim. Biophys. Acta *1436*, 279-298.

Stoll,L.L. and Spector,A.A. (1989) Interaction of platelet-activating factor with endothelial and vascular smooth muscle cells in coculture. J. Cell Physiol *139*, 253-261.

Strålin,P., Karlsson,K., Johansson,B.O., and Marklund,S.L. (1995) The interstitium of the human arterial wall contains very large amounts of extracellular superoxide dismutase. Arterioscler. Thromb. Vasc. Biol. *15*, 2032-2036.

Strauss,B.H., Robinson,R., Batchelor,W.B., Chisholm,R.J., Ravi,G., Natarajan,M.K., Logan,R.A., Mehta,S.R., Levy,D.E., Ezrin,A.M., and Keeley,F.W. (1996) In vivo collagen turnover following experimental balloon angioplasty injury and the role of matrix metalloproteinases. Circ. Res. *79*, 541-550.

Suzuki,J., Isobe,M., Morishita,R., Aoki,M., Horie,S., Okubo,Y., Kaneda,Y., Sawa,Y., Matsuda,H., Ogihara,T., and Sekiguchi,M. (1997) Prevention of graft coronary arteriosclerosis by antisense cdk2 kinase oligonucleotide. Nat Med *3*, 900-903.

Tardif,J.C., Cote,G., Lesperance,J., Bourassa,M., Lambert,J., Doucet,S., Bilodeau,L., Nattel,S., and de,G.P. (1997) Probucol and multivitamins in the prevention of restenosis after coronary angioplasty. Multivitamins and Probucol Study Group. N. Engl. J. Med. *337*, 365-372.

Tedgui,A. and Mallat,Z. (2001) Anti-inflammatory mechanisms in the vascular wall. Circ. Res. *88*, 877-887.

Tew,D.G., Southan,C., Rice,S.Q.J., Lawrence,G.M.P., Li,H.D., Boyd,H.F., Moores,K., Gloger,I.S., and Macphee,C.H. (1996) Purification, properties, sequencing, and cloning of a lipoprotein-associated, serine-dependent phospholipase involved in the oxidative modification of low-density lipoproteins. Arteriosclerosis Thrombosis and Vascular Biology *16*, 591-599.

Theilmeier,G., De,G.B., Van Veldhoven,P.P., Stengel,D., Michiels,C., Lox,M., Landeloos,M., Chapman,M.J., Ninio,E., Collen,D., Himpens,B., and Holvoet,P. (2000) HDL-associated PAF-AH reduces endothelial adhesiveness in apoE-/- mice. FASEB J. *14*, 2032-2039.

Thomas,C.E., Jackson,R.L., Ohlweiler,D.F., and Ku,G. (1994) Multiple lipid oxidation products in low density lipoproteins induce interleukin-1 beta release from human blood mononuclear cells. J. Lipid Res. *35*, 417-427.

Tjoelker,L.W. and Stafforini,D.M. (2000) Platelet-activating factor acetylhydrolases in health and disease. Biochim. Biophys. Acta *1488*, 102-123.

Tokumura,A., Kamiyasu,K., Takauchi,K., and Tsukatani,H. (1987) Evidence for existence of various homologues and analogues of platelet activating factor in a lipid extract of bovine brain. Biochem. Biophys. Res. Commun. *145*, 415-425.

Turpeinen,A.M., Alfthan,G., Valsta,L., Hietanen,E., Salonen,J.T., Schunk,H., Nyyssonen,K., and Mutanen,M. (1995) Plasma and lipoprotein lipid peroxidation in humans on sunflower and rapeseed oil diets. Lipids *30*, 485-492.

Turunen,M.P., Puhakka,H.L., Koponen,J.K., Hiltunen,M.O., Rutanen,J., Leppanen,O., Turunen,A.M., Narvanen,A., Newby,A.C., Baker,A.H., and Yla-Herttuala,S. (2002) Peptide-retargeted adenovirus encoding a tissue inhibitor of metalloproteinase-1 decreases restenosis after intravascular gene transfer. Mol. Ther. *6*, 306-312.

Turunen,P., Jalkanen,J., Heikura,T., Puhakka,H., Karppi,J., Nyyssonen,K., and Yla-Herttuala,S. (2004) Adenovirus-mediated gene transfer of Lp-PLA2 reduces LDL degradation and foam cell formation in vitro. J. Lipid Res. *45*, 1633-1639.

Vigna,E. and Naldini,L. (2000) Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy. J. Gene Med. *2*, 308-316.

Vogel,R.A., Corretti,M.C., and Gellman,J. (1998) Cholesterol, cholesterol lowering, and endothelial function. Prog. Cardiovasc. Dis. *41*, 117-136.

Vora,D.K., Fang,Z.T., Liva,S.M., Tyner,T.R., Parhami,F., Watson,A.D., Drake,T.A., Territo,M.C., and Berliner,J.A. (1997) Induction of P-selectin by oxidized lipoproteins. Separate effects on synthesis and surface expression. Circ. Res. *80*, 810-818.

Waksman,R. (2001) Vascular brachytherapy approval for clinical use. *2*, 1-2.

Waksman,R., Bhargava,B., Mintz,G.S., Mehran,R., Lansky,A.J., Satler,L.F., Pichard,A.D., Kent,K.M., and Leon,M.B. (2000) Late total occlusion after intracoronary brachytherapy for patients with in-stent restenosis. J. Am. Coll. Cardiol. *36*, 65-68.

Walsh,K., Smith,R.C., and Kim,H.S. (2000) Vascular cell apoptosis in remodeling, restenosis, and plaque rupture. Circ. Res. 2000. Aug. 4. ;87. (3.):184. -8. *87*, 184-188.

Wan,S., Yim,A.P., Johnson,J.L., Shukla,N., Angelini,G.D., Smith,F.C., Dashwood,M.R., and Jeremy,J.Y. (2004) The endothelin 1A receptor antagonist BSF 302146 is a potent inhibitor of neointimal and medial thickening in porcine saphenous vein-carotid artery interposition grafts. J. Thorac. Cardiovasc. Surg. *127*, 1317-1322.

Watson,A.D., Leitinger,N., Navab,M., Faull,K.F., Horkko,S., Witztum,J.L., Palinski,W., Schwenke,D., Salomon,R.G., Sha,W., Subbanagounder,G., Fogelman,A.M., and Berliner,J.A. (1997) Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. J. Biol. Chem. *272*, 13597-13607.

West,N., Guzik,T., Black,E., and Channon,K. (2001a) Enhanced superoxide production in experimental venous bypass graft intimal hyperplasia: role of NAD(P)H oxidase. Arterioscler. Thromb. Vasc. Biol. *21*, 189-194.

West,N.E., Qian,H., Guzik,T.J., Black,E., Cai,S., George,S.E., and Channon,K.M. (2001b) Nitric oxide synthase (nNOS) gene transfer modifies venous bypass graft remodeling: effects on vascular smooth muscle cell differentiation and superoxide production. Circulation *104*, 1526-1532.

Weyrich,A.S., McIntyre,T.M., McEver,R.P., Prescott,S.M., and Zimmerman,G.A. (1995) Monocyte tethering by P-selectin regulates monocyte chemotactic protein-1 and tumor necrosis factor-alpha secretion. Signal integration and NF-kappa B translocation. J. Clin. Invest *95*, 2297-2303.

Willerson,J.T. (2002) Systemic and local inflammation in patients with unstable atherosclerotic plaques. Prog. Cardiovasc. Dis. *44*, 469-478.

Williams,K.J. and Tabas,I. (1995) The response-to-retention hypothesis of early atherogenesis. Arterioscler. Thromb. Vasc. Biol. *15*, 551-561.

Witztum,J.L. and Steinberg,D. (1991) Role of oxidized low density lipoprotein in atherogenesis. J. Clin. Invest. *88*, 1785-1792.

Xu,Q., Zhang,Z., Davison,F., and Hu,Y. (2003) Circulating progenitor cells regenerate endothelium of vein graft atherosclerosis, which is diminished in ApoE-deficient mice. Circ. Res. *93*, e76-e86.

Yang,Y., Greenough,K., and Wilson,J.M. (1996a) Transient immune blockade prevents formation of neutralizing antibody to recombinant adenovirus and allows repeated gene transfer to mouse liver. Gene Ther. *3*, 412-420.

Yang,Z.Y., Simari,R.D., Perkins,N.D., San,H., Gordon,D., Nabel,G.J., and Nabel,E.G. (1996b) Role of the p21 cyclin-dependent kinase inhibitor in limiting intimal cell proliferation in response to arterial injury. Proc. Natl. Acad. Sci. U. S. A *93*, 7905-7910.

Ylä-Herttuala,S. (1994) Role of lipid and lipoprotein oxidation in the pathogenesis of atherosclerosis. Drugs of Today *30*, 507-514.

Ylä-Herttuala,S., Luoma,J., Viita,H., Hiltunen,T., Sisto,T., and Nikkari,T. (1995) Transfer of 15-lipoxygenase gene into rabbit iliac arteries results in the appearance of oxidation-specific lipid-protein adducts characteristic of oxidized low density lipoprotein. J. Clin. Invest. *95*, 2692-2698.

Ylä-Herttuala,S. and Martin,J.F. (2000) Cardiovascular gene therapy. Lancet *355*, 213-222.

Ylä-Herttuala,S., Palinski,W., Rosenfeld,M.E., Parthasarathy,S., Carew,T.E., Butler,S., Witztum,J.L., and Steinberg,D. (1989) Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. J. Clin. Invest. *84*, 1086-1095.

Zhu,N.L., Wu,L., Liu,P.X., Gordon,E.M., Anderson,W.F., Starnes,V.A., and Hall,F.L. (1997) Downregulation of cyclin G1 expression by retrovirus-mediated antisense gene transfer inhibits vascular smooth muscle cell proliferation and neointima formation. Circulation *96*, 628-635.

Zimmerman,G.A., McIntyre,T.M., Prescott,S.M., and Stafforini,D.M. (2002) The platelet-activating factor signaling system and its regulators in syndromes of inflammation and thrombosis. Crit Care Med. *30*, S294-S301.

Zorina,S.G. and Jaikirshan,J.K. (2002) Matrix metalloproteinases in Vascular remodeling and Atherogenesis: The Good, the Bad and the Ugly. Circ Res *90*, 251-262.

Zou,Y., Dietrich,H., Hu,Y., Metzler,B., Wick,G., and Xu,Q. (1998) Mouse model of venous bypass graft arteriosclerosis. Am. J. Pathol. *153*, 1301-1310.

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Adenovirus-mediated gene transfer of Lp-PLA_2 reduces LDL degradation and foam cell formation in vitro

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Abstract Oxidation of LDL generates biologically active platelet-activating factor (PAF)-like phospholipid derivatives, which have potent proinflammatory activity. These products are inactivated by lipoprotein-associated phospholipase A_2 (Lp-PLA_2), an enzyme capable of hydrolyzing PAF-like phospholipids. In this study, we generated an adenovirus (Ad) encoding human Lp-PLA_2 and injected 10^8, 10^9, and 10^{10} plaque-forming unit doses of Adlp-PLA_2 and control AdlacZ intra-arterially into rabbits to achieve overexpression of Lp-PLA_2 in liver and in vivo production of Lp-PLA_2-enriched LDL. As a result, LDL particles with 3-fold increased Lp-PLA_2 activity were produced with the highest virus dose. Increased Lp-PLA_2 activity in LDL particles decreased the degradation rate in RAW 264 macrophages after standard in vitro oxidation to 60–80% compared with LDL isolated from LacZ-transduced control rabbits. The decrease was proportional to the virus dose and Lp-PLA_2 activity. Lipid accumulation and foam cell formation in RAW 264 macrophages were also decreased when incubated with oxidized LDL containing the highest Lp-PLA_2 activity. Inhibition of the Lp-PLA_2 activity in the LDL particles led to an increase in lipid accumulation and foam cell formation. It is concluded that increased Lp-PLA_2 activity in LDL attenuates foam cell formation and decreases LDL oxidation and subsequent degradation in macrophages.—Turunen, P., J. Jalkanen, T. Heikura, H. Puhakka, J. Karppi, K. Nyyssönen, and S. Ylä-Herttuala. **Adenovirus-mediated gene transfer of Lp-PLA_2 reduces LDL degradation and foam cell formation in vitro. *J. Lipid Res.* 2004.** 45: **1633–1639.**

Supplementary key words lipoprotein-associated phospholipase A_2 • low density lipoprotein • macrophage

Oxidized low density lipoprotein (oxLDL) plays an important role in atherosclerosis. Uptake of oxLDL contributes to the formation of foam cells by arterial macrophages. OxLDL also plays other roles in atherogenesis, such as being cytotoxic and stimulating the migration of monocytes into the arterial wall (1). Oxidation of LDL is a free radical-mediated, autocatalytic process. Polyunsaturated fatty acids, which are present in LDL lipids, are main targets of the reactive forms of oxygen. Initial products of phospholipid oxidation are usually hydroperoxy derivatives, which give rise to a variety of aldehyde products (2). This fragmentation also leads to the formation of polar phospholipids containing short-chain acyl groups at the *sn*-2 position (3). These molecules serve as substrates for lipoprotein-associated phospholipase A_2 (Lp-PLA_2), also known as platelet-activating factor-acetylhydrolase (PAF-AH), which hydrolyzes them to lysophospholipids (4). Some of the biologic effects of oxLDL can be mimicked by phospholipids that are subjected to oxidation in vitro. The ability of oxidized phospholipids to induce monocyte binding can be completely abolished by Lp-PLA_2 (5).

Atherosclerosis has features of chronic inflammation, and oxLDL has been implicated as a factor involved in arterial wall inflammation (6). During LDL oxidation, biologically active PAF-like phospholipid derivatives are produced that may contribute to the proinflammatory properties of oxLDL (7). PAF levels are found to be higher in coronary arteries from patients with severe atherosclerosis, and antibodies to PAF are associated with borderline hypertension, early atherosclerosis, and metabolic syndrome (8–10). Because it is difficult to increase Lp-PLA_2 activity in LDL particles in vitro without the risk of causing alterations in lipoprotein structure and physiological properties, we generated adenoviruses encoding Lp-PLA_2 cDNA and injected these viruses intra-arterially into rabbits to achieve overexpression of Lp-PLA_2 in liver and in vivo production of LDL particles with increased Lp-PLA_2 activity to study the effects of Lp-PLA_2 on LDL

Abbreviations: Ad, adenovirus; ALAT, alanine aminotransferase; CRP, C-reactive protein; IL-6, interleukin-6; LPDS, lipoprotein-deficient serum; Lp-PLA_2, lipoprotein-associated phospholipase A_2; lyso-PC, lysophosphatidylcholine; oxLDL, oxidized low density lipoprotein; PAF-AH, platelet-activating factor-acetylhydrolase; pfu, plaque-forming units; SMC, smooth muscle cell; TBARS, thiobarbituric acid-reactive substance.

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degradation and foam cell formation in vitro. It was found that Lp-PLA_2 gene transfer led to an increased enzyme activity in isolated LDL particles, with potentially antiatherogenic effects on LDL oxidation, subsequent degradation, and decreased foam cell formation in RAW 264 macrophages in vitro.

METHODS

Materials

All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. (St. Louis, MO). Cell culture reagents were from Gibco BRL (Paisley, UK) unless otherwise stated. Cell lines were from ATCC (Manassas, VA), and chamber slides were from LabTek Brand, Nunc International (Roskilde, Denmark). ^{125}I was from Wallac Finland Oy (Helsinki, Finland). The PAF-AH assay kit was from Cayman Chemical (Ann Arbor, MI), and the Lp-PLA_2 inhibitor Pefabloc (4-[2-aminoethyl]benzosulfonylfluoride) was purchased from Roche Diagnostics (Mannheim, Germany).

Generation of adenoviruses

The Lp-PLA_2 adenoviruses were constructed and produced with the Adeno-X™ Expression System (Clontech, Palo Alto, CA), which is based on serotype 5 adenovirus. Human Lp-PLA_2 cDNA (11) was cloned into Adeno-X™ Viral DNA with cytomegalovirus immediate early promoter and bovine growth hormone poly(A). The recombinant Adeno-X™ DNA was packaged into adenoviruses by transfecting HEK 293 cells using Fugene 6 reagent (Boehringer Mannheim, Mannheim, Germany). Replication-deficient adenoviruses were produced in HEK 293 cells and purified by CsCl gradient centrifugation. The identity of the viruses was confirmed from viral DNA by PCR using human Lp-PLA_2-specific primers. The production of LacZ control adenoviruses has been previously described (12). Purified virus preparations were analyzed for the absence of toxicity, wild-type viruses, microbiological contaminants, and lipopolysaccharide as described (12).

Gene transfer in vitro

RAW 264 cells (ATCC) and rabbit aortic smooth muscle cells (SMCs) (13) were transduced with adenoviruses at multiplicities of infection of 100, 1,000, and 5,000. The medium was changed to Optimem (Gibco BRL) containing 0.5% lipoprotein-deficient serum (LPDS) and 1% penicillin streptomycin. The cells were grown for 48 h, and medium was collected and lyophilized for enzyme activity and immunoblot analyses. Total RNA was isolated for RT-PCR analysis using Trizol reagent (Gibco BRL).

Immunoblot analysis

Lyophilized medium of the adenovirus Lp-PLA_2 (Adlp-PLA_2)-transduced RAW 264 cells and SMCs was subjected to 12% SDS-PAGE. Samples were mixed with loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromphenol blue, and 10% glycerol) and incubated for 4 min at 95°C before application to the gel. The resolved proteins were blotted on Immobilon polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Lp-PLA_2 was detected with human PAF-AH polyclonal antiserum (dilution, 1:1,000) according to the manufacturer's instructions (Cayman Chemical). Human plasma PAF-AH was used as a positive control (Cayman Chemical).

Animal work

LDL was isolated for in vitro studies from 12 New Zealand White rabbits after the gene transfer. Fentanyl-fluanisone (0.3 ml/kg sc; Janssen Pharmaceutica, Beerse, Belgium) and midazolam (1.5 mg/kg im; Roche, Basel, Switzerland) were used for anesthesia. The gene transfer was done via common carotid artery with 5F introducer (Cordis Corp., Miami Lakes, FL) after 2 weeks of a 0.5% cholesterol diet. The doses of adenovirus (Adlp-PLA_2 and AdlacZ) were 10^8, 10^9, and 10^{10} plaque-forming units (pfu). As an additional control, a subgroup of rabbits (n = 3) were injected with physiological saline only. Serum (40–60 ml/rabbit) was collected for LDL isolation and enzyme activity measurements. Livers were removed for histological analyses. The animal protocol was approved by the Experimental Animal Committee of the University of Kuopio, Finland.

Isolation and modification of LDL

LDL was isolated from fasting serum of the transduced rabbits by ultracentrifugation (14) and radioiodinated with ^{125}I using Iodogen (Pierce Chemical Co., Rockford, IL) as an oxidizing agent (15) before standardized 18 h incubation with Cu^{2+} (20 μM) (14). Specific activities of the labeled LDLs were 250–500 cpm/ng protein. In one group, Lp-PLA_2 activity was irreversibly inhibited by 0.1 mM Pefabloc (16) at 37°C for 30 min before LDL iodination and oxidation. Dialysis steps were performed overnight in 0.9% NaCl and 0.01% EDTA, pH 7.4, at 4°C. After incubation, the medium was analyzed using agarose gel electrophoresis (Paragon Lipoprotein Electrophoresis Kit; Beckman, Namur, Belgium).

Analysis of blood samples

Serum and LDL Lp-PLA_2 activities were determined by using a commercially available assay kit according to the manufacturer's instructions (Cayman Chemical). The assay uses 2-thio-PAF, which serves as a substrate for Lp-PLA_2. Upon hydrolysis of the acetyl thioester bond by Lp-PLA_2, free thiols are detected using 5,5′-dithiobis-2-nitrobenzoic acid (Ellman's reagent). The absorbance is read at 414 nm over a period of time using an ELISA plate reader. Absorbance values were plotted as a function of time, and Lp-PLA_2 activity was calculated from the linear portion of the curve and expressed as nanomoles per milliliter per minute. The lipid peroxide content of LDL (150 μg) was estimated by measuring the thiobarbituric acid-reactive substance (TBARS) produced in terms of malondialdehyde (14). Conjugated diene formation was measured from plasma samples as described previously (17) and expressed as millimoles per mole of cholesterol. Blood plasma values of total cholesterol, alanine aminotransferase (ALAT), and C-reactive protein (CRP) were measured on day 7 using routine clinical chemistry assays at the Kuopio University Hospital Laboratory.

RT-PCR

For assessment of Lp-PLA_2 mRNA expression, RT-PCR was performed from liver tissue 7 days after the gene transfer and also from RAW 264 cells and SMCs at 48 h after virus transduction. Total RNA was isolated from the liver samples and from cell cultures after homogenization in Trizol reagent and treated with RQ1 RNase-free DNase (Promega, Madison, WI). Four micrograms of total RNA was reverse-transcribed using random hexamer primers (Promega) and M-MULV Reverse Transcriptase (New England Biolabs, Beverly, MA). cDNA was amplified by PCR using DyNAzyme™ II DNA Polymerase (Finnzymes, Espoo, Finland) and primers specific for human Lp-PLA_2 sequence as follows: forward, 5′-TGGAGCAACGGTTATTCAG-3′; reverse, 5′-TGGTTGTGTTAATGTTGGTCC-3′. The reaction was subjected

to 45 cycles of denaturing at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min. Extension in the final cycle was 7 min.

Histological analysis

Immunohistochemical staining for paraffin-embedded liver sections was performed for detection of Lp-PLA_2 protein expression after gene transfer. As a primary antibody, anti-human PAF-AH polyclonal antiserum was used (dilution, 1:500). Control immunostainings were conducted with sections from AdlacZ-transduced rabbits and also with Lp-PLA_2 sections without the primary antibody. Hematoxylin was used as a counter stain. The horseradish peroxidase system 3,3′-diaminobenzidine tetrahydrochloride-plus kit (DAB-Plus Substrate Kit; Zymed Laboratories, South San Francisco, CA) was used for signal detection according to the manufacturer's instructions.

Degradation assays and interleukin-6 measurements

Degradation assays were performed in RAW 264 cells in Optimem containing 10% LPDS with 10 μg/ml of the isolated, labeled, and oxLDL fractions (14). The medium was collected at different time points (3, 6, 12, and 24 h), and the amount of ^{125}I-labeled acid-soluble material in the medium (degradation) was determined. Values obtained from empty wells were subtracted before calculating the results. Protein concentrations from the RAW 264 cells and the LDL fractions were determined by the method of Lowry et al. (18). Interleukin-6 (IL-6) concentrations in the culture supernatants were determined with an ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Foam cell formation

RAW 264 cells were plated on chamber slides and incubated for 18 h with Optimem and 10% LPDS containing 100 μg/ml of the LDL fractions isolated from rabbits given different adenovirus doses (19). One Lp-PLA_2 group was treated with the Lp-PLA_2 inhibitor Pefabloc. After incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde, and stained with Oil Red O in 60% isopropanol, 0.4% dextrin, and hematoxylin. Foam cell formation was evaluated by light microscopy.

Statistics

All data are expressed as means ± SD. ANOVA followed by unpaired Student's *t*-test was used to evaluate statistical significances. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Transgene expression and the functionality of the Adlp-PLA_2 were verified at mRNA, protein, and enzyme activity levels in vitro and in vivo. Rabbit aortic SMCs and RAW 264 cells were transduced with Adlp-PLA_2. The total RNA was collected after 48 h for RT-PCR. The presence of Lp-PLA_2 transcript was detected in Adlp-PLA_2-transduced cells by using primers specific for human Lp-PLA_2 (**Fig. 1A**). Western blot analysis of lyophilized medium of the same cells showed in the Adlp-PLA_2-transduced cell supernatants but not in the untransduced control cell supernatants the presence of an ~65 kDa protein (Fig. 1B), which corresponds to the molecular mass identified for the glycosylated form of serum Lp-PLA_2 (11). The maximal Lp-PLA_2 enzyme activity in the lyophilized medium showed over 10-fold increased activity in SMCs and RAW 264 cells compared with the activity in the untransduced controls (Fig. 1C). In vivo, Adlp-PLA_2-transduced rabbit livers showed human Lp-PLA_2 mRNA expression in RT-PCR analysis 7 days after the gene transfer, but no signal was present in AdlacZ-transduced control livers (**Fig. 2A**). Immunostaining with anti-human PAF-AH antiserum showed protein expression in liver sections from Adlp-PLA_2-trans-

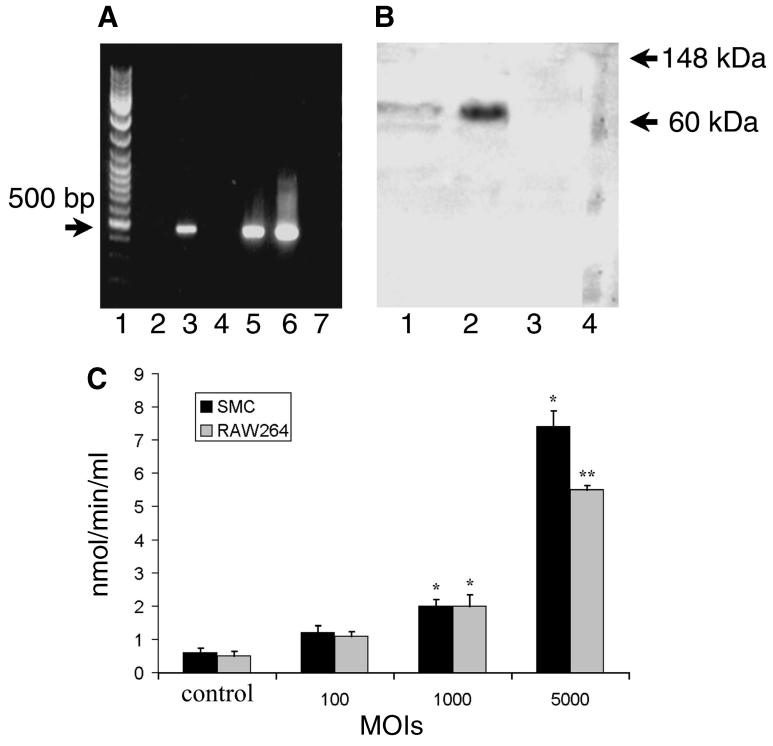

Fig. 1. Expression of human lipoprotein-associated phospholipase A_2 (Lp-PLA_2) after adenoviral gene transfer. A: Lp-PLA_2 mRNA was detected by RT-PCR from smooth muscle cell (SMC) and RAW 264 cell cultures 48 h after transduction with adenovirus Lp-PLA_2 (Adlp-PLA_2). Lane 1, ladder; lane 2, untransduced RAW 264 cells; lane 3, transduced RAW 264 cells; lane 4, untransduced SMCs; lane 5, transduced SMCs; lane 6, positive control (Lp-PLA_2 Adeno-X™ plasmid); lane 7, negative control. B: Western blot analysis of SMCs transduced with Adlp-PLA_2. Media from cells were collected after 48 h of incubation and lyophilized, and 20 ng of protein was electrophoresed on SDS-PAGE. Lane 1, medium from transduced SMCs; lane 2, positive control [purified human platelet-activating factor-acetylhydrolase (PAF-AH)]; lane 3, medium from untransduced SMCs; lane 4, molecular mass marker. Lp-PLA_2 was detected with human polyclonal PAF-AH antiserum as described in Methods. C: Lp-PLA_2 enzyme activities measured from media collected from SMCs and RAW 264 cells transduced with different multiplicities of infection (MOIs) of Lp-PLA_2. Media from untransduced cells were used as a control. All values are means ± SD of three determinations. ANOVA was followed by Student's *t*-test (* $P < 0.05$, ** $P < 0.002$).

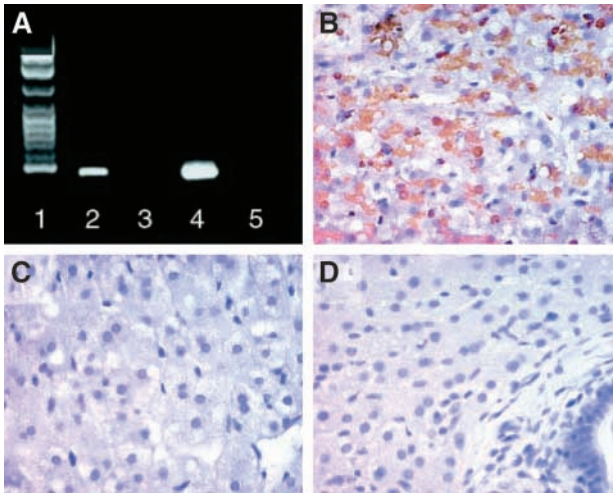

Fig. 2. Expression of human Lp-PLA_2 alter adenoviral gene transfer in vivo. A: Lp-PLA_2 mRNA was detected by RT-PCR from liver tissue 7 days after Lp-PLA_2 gene transfer. Lane 1, ladder; lane 2, liver from Adlp-PLA_2-transduced rabbits; lane 3, liver from AdlacZ-transduced rabbits; lane 4, positive control (Lp-PLA_2 Adeno-X™ plasmid); lane 5, negative control. B: Immunostaining with human polyclonal PAF-AH antiserum (dilution, 1:500) shows positive staining 7 days after gene transfer in liver sections from Adlp-PLA_2-transduced [10^{10} plaque-forming units (pfu)] rabbits. C: No immunostaining is seen in liver in LacZ-transduced rabbits. D: Nonimmune control for the immunostainings. Magnification, ×40.

duced rabbits but not in the control LacZ rabbits (Fig. 2, B–D). The gene transfer also led to increased Lp-PLA_2 activity in serum: at an adenovirus dose of 10^8 pfu (n = 4), the activities were 49 ± 1.4 nmol/min/ml in the Lp-PLA_2 group and 42 ± 2.8 nmol/min/ml in the LacZ group; at a dose of 10^9 pfu (n = 4), the activities were 51 ± 4.9 nmol/min/ml in the Lp-PLA_2 group and 38 ± 4.2 nmol/min/ml in the LacZ group; and at a dose 10^{10} pfu (n = 4), the activities were 63 ± 1.4 nmol/min/ml in the Lp-PLA_2 group and 38 ± 2.8 nmol/min/ml in the LacZ group. Lp-PLA_2 activity was also measured from isolated rabbit LDL, and an aliquot of the LDL from each dose group was treated with Pefabloc. Lp-PLA_2 inhibitor treatment irreversibly inhibited the Lp-PLA_2 activity in rabbit LDL (**Fig. 3A**).

Blood plasma values (means ± SEM) of total cholesterol, CRP, and ALAT at 7 days after the gene transfer are shown in Fig. 3, B–D. There were no statistically significant differences between the study groups. In addition, TBARS and diene measurements from plasma and isolated LDLs did not show any statistically significant differences between the groups (data not shown). Incubation of native LDL or oxLDL with RAW 264 cells for 18 and 48 h did not lead to any increased IL-6 production by the cells, indicating no major inflammatory activation of RAW 264 macrophages during the experiments (data not shown).

The effect of the increased Lp-PLA_2 activity on LDL oxidation and subsequent degradation in macrophages was analyzed using LDLs isolated from rabbits given different doses of Adlp-PLA_2. Agarose gel electrophoresis showed a slight decrease in the migration of ^{125}I-LDL isolated from the Adlp-PLA_2 group (10^{10} pfu) compared with the migration of control LacZ ^{125}I-LDL and ^{125}I-LDL treated with the Lp-PLA_2 inhibitor Pefabloc (**Fig. 4**). Degradation in RAW 264 macrophages of the isolated LDL fractions subjected to standardized oxidation was followed at different time points (3, 6, 12, and 24 h). Increased Lp-PLA_2 content in LDL particles decreased the degradation of LDL after oxidation to 60–87% of the control LacZ LDL degradation. The values at different time points were 87% for 3 h, 60% for 6 h, 77% for 12 h, and 74% for 24 h (data not shown). Next, we studied the degradation of LDL isolated from rabbits given different adenovirus doses. Enzyme activities of the isolated LDLs are shown in Fig. 3A. The cells were incubated with medium containing 10 μg/ml LDL for 6 h. As a result, the increased Lp-PLA_2 activity in the rabbit LDLs decreased the degradation of LDL after oxidation to 63–87% of the LacZ control LDL values (**Fig. 5**). Inhibition of the Lp-PLA_2 activity by Pefabloc led to a 2-fold increase in the degradation compared with the LacZ control LDL. Inhibition of the degradation was most effective with LDL containing the highest level of Lp-PLA_2 activity. To test whether Lp-PLA_2 could inhibit the uptake of larger quantities of oxLDL, we tested the effect of increased Lp-PLA_2 activity on foam cell formation in RAW 264 macrophages, incubating the cells for 18 h with 100 μg/ml LDLs isolated from the transduced rabbits and subjected to standardized oxidation. The lipid accumulation in RAW 264 macrophages and foam cell formation were decreased when incubated with oxLDL containing the highest Lp-PLA_2 activity (**Fig. 6**). When Lp-PLA_2 was irreversibly inhibited in the LDL particle, it led to an increase in lipid accumulation and foam cell formation compared with that in the Lp-PLA_2 and LacZ control LDL groups (Fig. 6).

DISCUSSION

Lp-PLA_2 is a member of the phospholipase A_2 superfamily, which consists of a large number of enzymes defined by their ability to catalyze the hydrolysis of the *sn*-2 ester bond in phospholipids (20). The biological role of Lp-PLA_2 is to hydrolyze PAF and other polar phospholipids with a short, oxidized acyl chain in the *sn*-2 position of glycerol (21). The specificity of the enzyme for this type of acyl group ensures that phospholipid components of cellular membranes and lipoproteins remain intact while products of oxidation and fragmentation are hydrolyzed. Lp-PLA_2 is a hydrophobic protein, and in plasma two-thirds of the enzyme activity is associated with LDL, the rest of the activity being associated mainly with HDL (22). On the other hand, less than 1% of the LDL particles contain Lp-PLA_2 (23). Thus, even a minor increase in Lp-PLA_2 content could have important effects on the properties of LDL. Because it is difficult to get hydrophobic proteins in LDL in a test tube, we wanted to direct overexpression of Lp-PLA_2 into the liver, where lipoprotein particles are produced. This was achieved with adenovirus-mediated gene transfer, because adenovirus given via a systemic route is known to lead to strong transgene expression in the liver

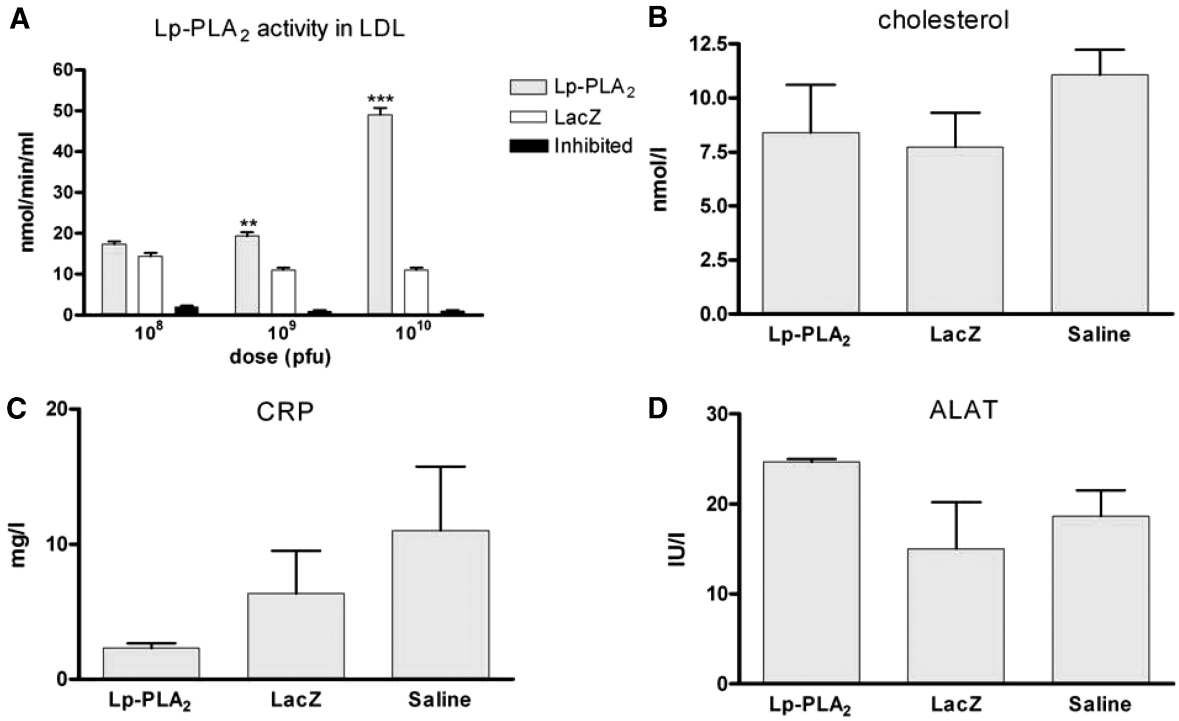

Fig. 3. Lp-PLA_2 activity in isolated LDL and analysis of plasma samples of the donor rabbits. A: Lp-PLA_2 activity in LDL particles isolated from rabbits treated with 10^8, 10^9, and 10^{10} pfu adenoviruses. An aliquot of LDL at each dose level was treated with Lp-PLA_2 inhibitor (0.1 mM Pefabloc for 30 min at 37°C), which irreversibly destroys Lp-PLA_2 enzyme activity in the particle. B–D: Total cholesterol (B), CRP (C), and ALAT (D) 7 days after the gene transfer (10^{10} pfu). All values are means ± SD of three determinations. ANOVA was followed by Student's *t*-test (Lp-PLA_2 vs. LacZ, ** $P < 0.002$, *** $P < 0.0001$).

(24). The purpose of the study was not to evaluate the effect of adenovirus-mediated Lp-PLA_2 gene transfer on atherogenesis in rabbits per se, because gene expression achieved in large animals by adenovirus only lasts for 1–2 weeks (24). Rather, the main goal was to achieve a short-term, effective Lp-PLA_2 overexpression in the liver for the production of LDLs with increased Lp-PLA_2 activity for in vitro studies. We considered these in vitro studies essential before trying any antiatherosclerotic gene transfer protocols in rabbits that would require the construction of alternative gene transfer vectors (25).

LDL oxidation plays an important role in atherogenesis (1). Oxidation of LDL is a multistep process in which the peroxidation of polyunsaturated fatty acids leads to the accumulation of lipid peroxidation products in the lipoprotein particle (so-called minimally modified LDL), followed by propagation of the lipid peroxidation, modification of apolipoprotein B, and accumulation of lysophosphatidylcholine (lyso-PC) in the LDL (26). It has been demonstrated that PAF-like lipids are also generated during LDL oxidation (3, 5). These lipids mimic PAF and can activate many types of cells via the PAF receptor and cause the stimulation of platelet aggregation, leukocyte activation and adhesion to endothelium, increased vascular permeability, monocyte activation, and production of superoxide anion by macrophages (27). The formation of PAF-like lipids is an important part of the biological activity of minimally modified LDL, whereas their role in fully ox-LDL remains unknown. Extensive oxidation of LDL creates particles that are metabolized in macrophages by scavenger receptors (28), which leads to the accumulation of intracellular cholesterol (1). In this study, LDL degradation and foam cell formation were used as biological indicators of the effects of increased Lp-PLA_2 activity. However, it should be kept in mind that both of these methods measure late stages in the sequence of LDL oxidation and require extensive modification of both apolipoprotein B-100 and lipid components of the studied LDL fractions. Nevertheless, we consider these analyzes important surrogate markers of LDL atherogenicity in vivo, because in-

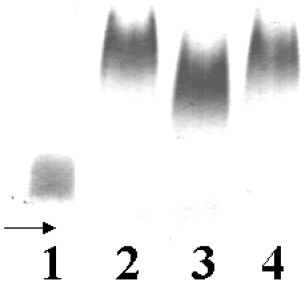

Fig. 4. ^{125}I-LDL isolated from rabbits given 10^{10} pfu of Lp-PLA_2 or LacZ adenovirus was incubated overnight with 20 μM Cu^{2+} and agarose gel electrophoresis was performed. Lane 1, native ^{125}I-LDL; lane 2, ^{125}I-oxidized low density lipoprotein (^{125}I-oxLDL; Pefabloc inhibition); lane 3, ^{125}I-oxLDL (Lp-PLA_2); lane 4, ^{125}I-oxLDL (LacZ). The arrow indicates the point of application.

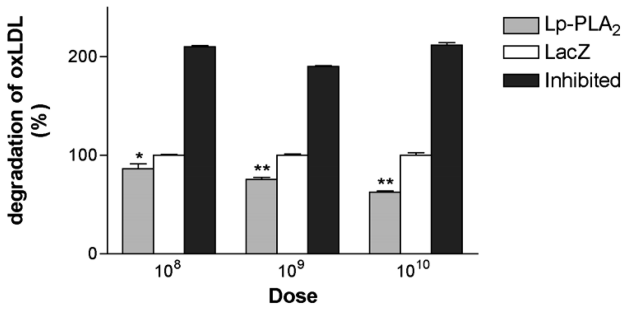

Fig. 5. Effect of Lp-PLA_2 on the degradation of oxLDL. RAW 264 cells were incubated for 6 h with 10 μg/ml ^{125}I oxLDL isolated from rabbits given different doses of adenovirus. An aliquot of LDL at each dose level was treated with 0.1 mM Pefabloc. Inhibition of the Lp-PLA_2 activity led to a 2-fold increase in degradation compared with that in the LacZ control LDL (marked as 100%). Inhibition of the degradation was most effective (63%) with LDL containing the highest level of Lp-PLA_2 activity. Absolute values of degraded oxLDL (ng/mg cell protein) in Lp-PLA_2 groups were as follows: 729 ± 66 (10^8 pfu), 632 ± 30 (10^9 pfu), and 429 ± 17.7 (10^{10} pfu); values in LacZ groups were 843 ± 13.6 (10^8 pfu), 837 ± 18.1 (10^9 pfu), and 683 ± 30.1 (10^{10} pfu); values in Pefabloc groups were 1,771 ± 18.6 (10^8 pfu), 1,592 ± 15.2 (10^9 pfu), and 1,444 ± 33.2 (10^{10} pfu). ANOVA was followed by Student's *t*-test (* $P < 0.05$, ** $P < 0.002$ for Lp-PLA_2 vs. LacZ). Degradation in LacZ medium is presented as 100% of degradation.

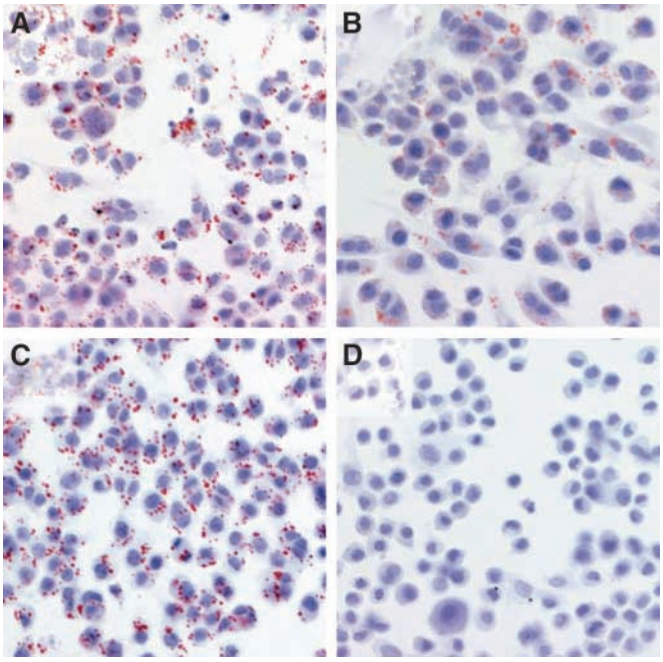

Fig. 6. Prevention of foam cell formation by Lp-PLA_2 in LDL particles. RAW 264 cells were incubated for 18 h with 100 μg/ml oxLDL. A: Foam cell formation with rabbit oxLDL from the LacZ control group. B: Lipid accumulation was decreased when cells were incubated with rabbit oxLDL containing the highest Lp-PLA_2 activity. C: When Lp-PLA_2 activity was inhibited with 0.1 mM Pefabloc, foam cell formation was markedly increased. D: Control cells incubated without LDL. Oil Red O staining is shown.

creased intracellular cholesterol accumulation is the hallmark of early human atherosclerotic lesions (1, 6).

Protective effects of Lp-PLA_2 on the atherogenic properties of LDL are most likely related to its ability to destroy oxidatively fragmented phospholipids, which are one class of ligands on oxLDL for macrophage scavenger receptor recognition (29). Reduction of these substances can also prevent the subsequent formation of fully oxLDL and uptake by macrophages. On the other hand, lyso-PC accumulating in fully oxLDL has several proatherogenic effects (30, 31). Thus, Lp-PLA_2 can potentially affect the LDL oxidation process in both the early and late phases by reducing bioactive PAF-like lipids in minimally modified LDL and causing lyso-PC accumulation in the lipoprotein particle. However, it is important to note that lyso-PC is water soluble and can diffuse out from LDL. In addition, some antiatherogenic actions of lyso-PC have also been reported, for example, the promotion of cholesterol efflux from macrophage foam cells (32). Therefore, it is possible that while protecting LDL particles from becoming oxidized, Lp-PLA_2 activity may lead to enhanced production of lyso-PC, which has multiple effects on atherogenesis. Recently, it was shown that inhibition of Lp-PLA_2 diminished the toxicity and apoptosis induced by fully oxLDL and that this protection was apparent only after a prolonged incubation (33). It has also been shown that HDL-associated Lp-PLA_2 activity prevents the oxidation of atherogenic lipoproteins and macrophage homing into apolipoprotein $E^{-/-}$ mouse lesions (34).

When interpreting these results, it should be remembered that we have expressed human Lp-PLA_2 in rabbits and that binding of the human enzyme to rabbit LDL may not fully resemble the human situation. Also, while preparing radiolabeled oxLDL for degradation studies, lipoprotein preparations have been dialyzed, and this could have reduced the lyso-PC content in the lipoprotein particles, modifying their biological properties. Thus, these results may not fully reflect the in vivo situation regarding the effects of Lp-PLA_2 on atherogenesis. Lastly, the inhibitor used for the study is not absolutely specific for Lp-PLA_2 and may have additional unknown effects on LDL oxidation and lipid uptake by macrophages.

In the context of atherosclerosis, the role of Lp-PLA_2 is still somewhat unclear; indeed, Lp-PLA_2 is considered to have a dual role: one that is anti-inflammatory (35) and one that is proinflammatory as a result of the generation of lyso-PC, which is an abundant component of oxLDL (30). Increased Lp-PLA_2 expression and activity have been demonstrated in human and rabbit atherosclerotic lesions (36). However, it remains unclear whether Lp-PLA_2 contributes to the progression of human lesions, and definitive conclusions about the proatherogenic and antiatherogenic roles of Lp-PLA_2 activity and its inhibition can only be obtained from prospective human intervention studies. Our results show that when subjected to in vitro oxidation, increased levels of Lp-PLA_2 activity in LDL reduce subsequent lipoprotein degradation and foam cell formation in macrophages, which suggest that in the early fatty streaks Lp-PLA_2 may have antiatherogenic effects by reducing proinflammatory changes and lipid uptake in lesion macrophages.

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REFERENCES

1. Steinberg, D. 2002. Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime. *Nat. Med.* **8:** 1211–1217.
2. Steinbrecher, U. P. 1987. Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. *J. Biol. Chem.* **262:** 3603–3608.
3. Tokumura, A., M. Toujima, Y. Yoshioka, and K. Fukuzawa. 1996. Lipid peroxidation in low density lipoproteins from human plasma and egg yolk promotes accumulation of 1-acyl analogues of platelet-activating factor-like lipids. *Lipids.* **31:** 1251–1258.
4. Stremler, K. E., D. M. Stafforini, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 1989. An oxidized derivative of phosphatidylcholine is a substrate for the platelet-activating factor acetylhydrolase from human plasma. *J. Biol. Chem.* **264:** 5331–5334.
5. Watson, A. D., M. Navab, S. Y. Hama, A. Sevanian, S. M. Prescott, D. M. Stafforini, T. M. McIntyre, B. N. Du, A. M. Fogelman, and J. A. Berliner. 1995. Effect of platelet activating factor-acetylhydrolase on the formation and action of minimally oxidized low density lipoprotein. *J. Clin. Invest.* **95:** 774–782.
6. Ross, R. 1999. Mechanisms of disease: atherosclerosis—an inflammatory disease. *N. Engl. J. Med.* **340:** 115–126.
7. Tjoelker, L. W., C. Wilder, C. Eberhardt, D. M. Stafforini, G. Dietsch, B. Schimpf, S. Hooper, H. Le Trong, L. S. Cousens, G. A. Zimmerman, Y. Yamada, T. M. McIntyre, S. M. Prescott, and P. W. Gray. 1995. Anti-inflammatory properties of a platelet-activating factor acetylhydrolase. *Nature.* **374:** 549–553.
8. Frostegard, J., Y. H. Huang, J. Ronnelid, and L. Schafer-Elinder. 1997. Platelet-activating factor and oxidized LDL induce immune activation by a common mechanism. *Arterioscler. Thromb. Vasc. Biol.* **17:** 963–968.
9. Mueller, H. W., C. A. Haught, J. M. McNatt, K. Cui, S. J. Gaskell, D. A. Johnston, and J. T. Willerson. 1995. Measurement of platelet-activating factor in a canine model of coronary thrombosis and in endarterectomy samples from patients with advanced coronary artery disease. *Circ. Res.* **77:** 54–63.
10. Wu, R., C. Lemne, U. de Faire, and J. Frostegard. 1999. Antibodies to platelet-activating factor are associated with borderline hypertension, early atherosclerosis and the metabolic syndrome. *J. Intern. Med.* **246:** 389–397.
11. Tew, D. G., C. Southan, S. Q. J. Rice, G. M. P. Lawrence, H. D. Li, H. F. Boyd, K. Moores, I. S. Gloger, and C. H. Macphee. 1996. Purification, properties, sequencing, and cloning of a lipoprotein-associated, serine-dependent phospholipase involved in the oxidative modification of low-density lipoproteins. *Arterioscler. Thromb. Vasc. Biol.* **16:** 591–599.
12. Laitinen, M., K. Mäkinen, H. Manninen, P. Matsi, M. Kossila, R. S. Agrawal, T. Pakkanen, J. S. Luoma, H. Viita, J. Hartikainen, E. Alhava, M. Laakso, and S. Yla-Herttuala. 1998. Adenovirus-mediated gene transfer to lower limb artery of patients with chronic critical leg ischemia. *Hum. Gene Ther.* **9:** 1481–1486.
13. Ylä-Herttuala, S., J. Luoma, H. Viita, T. Hiltunen, T. Sisto, and T. Nikkari. 1995. Transfer of 15-lipoxygenase gene into rabbit iliac arteries results in the appearance of oxidation-specific lipid-protein adducts characteristic of oxidized low density lipoprotein. *J. Clin. Invest.* **95:** 2692–2698.
14. Ylä-Herttuala, S., W. Palinski, M. E. Rosenfeld, S. Parthasarathy, T. E. Carew, S. Butler, J. L. Witztum, and D. Steinberg. 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.* **84:** 1086–1095.
15. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–221.
16. Dentan, C., A. D. Tselepis, M. J. Chapman, and E. Ninio. 1996. Pefabloc, 4-[2-aminoethyl]benzenesulfonyl fluoride, is a new, potent nontoxic and irreversible inhibitor of PAF-degrading acetylhydrolase. *Biochim. Biophys. Acta.* **1299:** 353–357.
17. Turpeinen, A. M., G. Alfthan, L. Valsta, E. Hietanen, J. T. Salonen, H. Schunk, K. Nyyssonen, and M. Mutanen. 1995. Plasma and lipoprotein lipid peroxidation in humans on sunflower and rapeseed oil diets. *Lipids.* **30:** 485–492.
18. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
19. Laukkanen, J., P. Lehtolainen, P. J. Gough, D. R. Greaves, S. Gordon, and S. Ylä-Herttuala. 2000. Adenovirus-mediated gene transfer of a secreted form of human macrophage scavenger receptor inhibits modified low-density lipoprotein degradation and foam-cell formation in macrophages. *Circulation.* **101:** 1091–1096.
20. Six, D. A., and E. A. Dennis. 2000. The expanding superfamily of phospholipase A(2) enzymes: classification and characterization. *Biochim. Biophys. Acta.* **1488:** 1–19.
21. Farr, R. S., C. P. Cox, M. L. Wardlow, and R. Jorgensen. 1980. Preliminary studies of an acid-labile factor (ALF) in human sera that inactivates platelet-activating factor (PAF). *Clin. Immunol. Immunopathol.* **15:** 318–330.
22. Stafforini, D. M., S. M. Prescott, and T. M. McIntyre. 1987. Human plasma platelet-activating factor acetylhydrolase. Purification and properties. *J. Biol. Chem.* **262:** 4223–4230.
23. Stafforini, D. M., G. A. Zimmerman, T. M. McIntyre, and S. M. Prescott. 1992. The platelet-activating factor acetylhydrolase from human plasma prevents oxidative modification of low-density lipoprotein. *Trans. Assoc. Am. Physicians.* **105:** 44–63.
24. Hiltunen, M. O., M. P. Turunen, A. M. Turunen, T. T. Rissanen, M. Laitinen, V. M. Kosma, and S. Ylä-Herttuala. 2000. Biodistribution of adenoviral vector to nontarget tissues after local in vivo gene transfer to arterial wall using intravascular and periadventitial gene delivery methods. *FASEB J.* **14:** 2230–2236.
25. Ylä-Herttuala, S., and J. F. Martin. 2000. Cardiovascular gene therapy. *Lancet.* **355:** 213–222.
26. Binder, C. J., M. K. Chang, P. X. Shaw, Y. I. Miller, K. Hartvigsen, A. Dewan, and J. L. Witztum. 2002. Innate and acquired immunity in atherogenesis. *Nat. Med.* **8:** 1218–1226.
27. Smiley, P. L., K. E. Stremler, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 1991. Oxidatively fragmented phosphatidylcholines activate human neutrophils through the receptor for platelet-activating factor. *J. Biol. Chem.* **266:** 11104–11110.
28. Ylä-Herttuala, S., M. E. Rosenfeld, S. Parthasarathy, E. Sigal, T. Särkioja, J. L. Witztum, and D. Steinberg. 1991. Gene expression in macrophage-rich human atherosclerotic lesions. 15-Lipoxygenase and acetyl low density lipoprotein receptor messenger RNA colocalize with oxidation specific lipid-protein adducts. *J. Clin. Invest.* **87:** 1146–1152.
29. Horkko, S., D. A. Bird, E. Miller, H. Itabe, N. Leitinger, G. Subbanagounder, J. A. Berliner, P. Friedman, E. A. Dennis, L. K. Curtiss, W. Palinski, and J. L. Witztum. 1999. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *J. Clin. Invest.* **103:** 117–128.
30. Quinn, M. T., S. Parthasarathy, and D. Steinberg. 1988. Lysophosphatidylcholine: a chemotactic factor for human monocytes and its potential role in atherogenesis. *Proc. Natl. Acad. Sci. USA.* **85:** 2805–2809.
31. Carson, M. J., and D. Lo. 2001. Immunology. The push-me pull-you of T cell activation. *Science.* **293:** 618–619.
32. Hara, S., T. Shike, N. Takasu, and T. Mizui. 1997. Lysophosphatidylcholine promotes cholesterol efflux from mouse macrophage foam cells. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1258–1266.
33. Carpenter, K. L., I. F. Dennis, I. R. Challis, D. P. Osborn, C. H. Macphee, D. S. Leake, M. J. Arends, and M. J. Mitchinson. 2001. Inhibition of lipoprotein-associated phospholipase A2 diminishes the death-inducing effects of oxidised LDL on human monocyte-macrophages. *FEBS Lett.* **505:** 357–363.
34. Theilmeier, G., B. De Geest, P. P. Van Veldhoven, D. Stengel, C. Michiels, M. Lox, M. Landeloos, M. J. Chapman, E. Ninio, D. Collen, B. Himpens, and P. Holvoet. 2000. HDL-associated PAF-AH reduces endothelial adhesiveness in apoE−/− mice. *FASEB J.* **14:** 2032–2039.
35. Tjoelker, L. W., and D. M. Stafforini. 2000. Platelet-activating factor acetylhydrolases in health and disease. *Biochim. Biophys. Acta.* **1488:** 102–123.
36. Häkkinen, T., J. S. Luoma, M. O. Hiltunen, C. H. Macphee, K. J. Milliner, L. Patel, S. Q. Rice, D. G. Tew, K. Karkola, and S. Yla-Herttuala. 1999. Lipoprotein-associated phospholipase A(2), platelet-activating factor acetylhydrolase, is expressed by macrophages in human and rabbit atherosclerotic lesions. *Arterioscler. Thromb. Vasc. Biol.* **19:** 2909–2917.

II

Intravascular adenovirus-mediated lipoprotein-associated phospholipase A_2 gene transfer reduces neointima formation in balloon-denuded rabbit aorta

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Intravascular adenovirus-mediated lipoprotein-associated phospholipase A_2 gene transfer reduces neointima formation in balloon-denuded rabbit aorta

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Abstract

Postangioplasty restenosis is a multifactorial process and involves mechanisms such as inflammation and stimulation of the expression of growth factors. Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) can modify inflammatory responses by hydrolyzing phospholipids with shortened and/or oxidized *sn*-2 residues. In this study, we tested a hypothesis that adenovirus-mediated Lp-PLA_2 gene transfer can reduce restenosis in rabbits.

Aortas of cholesterol-fed NZW rabbits were balloon-denuded and intra-arterial gene transfer was performed using Dispatch catheter with Lp-PLA_2 or LacZ adenoviruses (1.15×10^{10} pfu). Intima/media ratio (I/M), histology and cell proliferation were analyzed. Two weeks after the gene transfer I/M in the LacZ-transduced control group was 0.45 ± 0.05 but Lp-PLA_2 gene transfer reduced I/M to 0.25 ± 0.03. At four weeks time point I/M in the Lp-PLA_2 group (0.34 ± 0.05) was also lower than in the LacZ group (0.53 ± 0.06). Plasma Lp-PLA_2 activity was increased in the Lp-PLA_2 group (48.2 ± 4.2) as compared to the LacZ group (33.6 ± 3.51) at two weeks time point. Transgene expression was detected in the arterial wall two and four weeks after the procedure. Apoptosis was higher in the control vessels than in the Lp-PLA_2 group at two weeks time point.

In conclusion, local adenovirus-mediated Lp-PLA_2 gene transfer resulted in a significant reduction in neointima formation in balloon-denuded rabbit aorta and may be useful for the prevention of restenosis after arterial manipulations.

Keywords: Lipoprotein-associated phospholipase A_2; Adenovirus; Restenosis; Gene transfer

1. Introduction

Restenosis is an obstruction of a blood vessel due to tissue growth and negative remodeling at the site of angioplasty or arterial manipulation. About 20% of patients suffer from restenosis within six months after angioplasty and a repeated revascularization procedure may be needed [1]. Restenosis is a multifactorial process initiated by vessel trauma and involves mechanisms such as inflammation, secretion of growth factors and platelet activation [2–5]. Even though drug-eluting stents have improved the outcome of angioplasty there is still a clear need to develop new local therapies for the prevention and treatment of restenosis after arterial manipulations.

Platelet activating factor (PAF) is an important lipid mediator of inflammation that exhibits a wide range of biological activities [6]. PAF and PAF-like oxidized phospholipids are inactivated by lipoprotein associated phospholipase A_2 (Lp-PLA_2), also called as PAF-acetylhydrolase, which hydrolyzes phospholipids with shortened or/and oxidized *sn*-2 residues [7]. It has been demonstrated that angioplasty is accompa-

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nied by platelet and leukocyte activation, as well as by enhanced platelet-leukocyte adhesion which are involved in the inflammatory response after injury [8]. PAF is produced by a variety of cells such as monocytes/macrophages, platelets and endothelial cells after stimulation by inflammatory mediators [9]. This leads to the release of several biologically active agents which can induce smooth muscle cell proliferation [10]. PAF synthesized by endothelial cells is exposed on the cell surface and promotes leukocyte adhesion on the endothelium [11]. PAF-like phospholipids can also be formed within the vessel wall from membrane phospholipids during oxidative stress. Balloon denudation immediately increases superoxide anion (O_2^-) production [12] suggesting the presence of strong oxidant stress at the site of injury and formation of PAF-like and oxidized phospholipids. Accordingly, antioxidants have been proposed as a potential treatment for restenosis [13] and extracellular superoxide dismutase (EC-SOD) gene transfer reduces restenosis after balloon denudation in rabbits [14]. Thus, Lp-PLA_2 could potentially have both anti-inflammatory and antirestenotic activity.

In this study, we analyzed the effects of adenovirus-mediated Lp-PLA_2 gene transfer on neointimal formation in a rabbit restenosis model. Gene transfer was made locally at the site of injury with Dispatch catheter and it was found that gene transfer reduced intimal thickening in rabbit aorta.

2. Materials and methods

2.1. Materials

All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. PAF-AH assay kit was from Cayman Chemical. Plasma total cholesterol and triglycerides were measured by Cholesterin CHOD-PAP-kit (Roche). C-reactive protein (CRP) was measured by QuickRead CRP system (Orion Diagnostica) and aspartyl aminotransferase (ASAT) was measured at the Kuopio University Hospital laboratory. Apoptosis was detected with an ApopTag kit (Intergen).

2.2. Generation of adenoviruses

The Lp-PLA_2 adenoviruses were constructed and produced with the Adeno-X^{TM} Expression System (Clontech), which is based on serotype 5 adenovirus. Human Lp-PLA_2 cDNA [15] was cloned into Adeno-X-Viral DNA with CMV immediate early promoter and bovine growth hormone polyA. The recombinant Adeno-X DNA was packaged into infectious adenoviruses by transfecting HEK 293 cells using Fugene 6 reagent (Boehringer-Mannheim). Before preparation of high-titer viral stocks by CsCl gradient centrifugation, the identity of the virus was confirmed from viral DNA by PCR using human Lp-PLA_2 specific primers. The production of LacZ control adenoviruses has been described [16]. Purified viruses were analysed for the absence of toxicity, wild-type viruses, microbiological contaminants and lipopolysaccharide as described [16]. Functionality of the AdLp-PLA_2 was verified at mRNA, protein and enzyme activity levels in vitro [17].

2.3. Animal experiments

Thirty-two New Zealand White male rabbits were fed 0.25% cholesterol diet for two weeks prior the experiments in order to increase serum cholesterol levels and were randomly divided into four groups. Groups one ($n = 7$) and three ($n = 7$) had gene transfer with Lp-PLA_2 adenoviruses and groups two ($n = 8$) and four ($n = 10$) had gene transfer with LacZ adenoviruses. Gene transfer was performed three days after the balloon denudation [18]. The whole aorta was denuded twice with a 3.0F arterial embolectomy catheter (Sorin Biomedical). Three days later, the gene transfer was performed with a 3.0F drug delivery catheter (Dispatch catheter, Boston Scientific), which allows continuous blood flow during transduction [19]. Under fluoroscopic control, the catheter was positioned caudal to the left renal artery in a segment free of side branches. A virus titer of 1.15×10^{10} pfu was used in the final volume of 2 ml in 0.9% saline, and the gene transfer was performed at 6 atm pressure for 10 min (0.2 ml/min). Animals in groups one and two were sacrificed two weeks after the gene transfer and in groups three and four weeks after the gene transfer. Serum samples were collected before gene transfer and at time points 3, 7, 14 and 28 days after the gene transfer. Tissue samples from liver, spleen, lungs and kidneys were collected to determine the biodistribution of adenovirus. All studies were approved by the Experimental Animal Committee of the University of Kuopio.

2.4. Histology

Three hours before death animals were injected with 50 mg of bromodeoxyuridine (BrdU) dissolved in 40% ethanol. After death, the transduced segment was removed, flushed gently with saline, and divided into four equal parts. The proximal part was snap-frozen in liquid nitrogen and stored at −70 °C. The next part was immersion-fixed in 4% paraformaldehyde/15% sucrose (pH 7.4) for 4 h, rinsed in 15% sucrose (pH 7.4) overnight, and embedded in paraffin. The medial part was fixed in 4% paraformaldehyde/PBS (pH 7.4) for 10 min, rinsed in PBS, embedded in OCT compound (Miles), and stored at −70 °C. The fourth part for BrdU sections was fixed in 70% ethanol overnight and embedded in paraffin [18]. Tissue samples for biodistribution analysis were collected, flushed with saline and divided into two parts. One part was snap frozen in liquid nitrogen and stored at −70 °C. The second part was immersion fixed in 4% paraformaldehyde/15% sucrose (pH 7.4) for 4 h, rinsed in 15% sucrose (pH 7.4) overnight and embedded in paraffin. Gene transfer efficiency was determined by x-gal staining as described [14]. Neointima formation was measured after hematoxylin-eosin staining. Immunohistochemical stainings

were performed for the detection of macrophages (RAM-11; DAKO, dilution 1:50), endothelium (CD31; DAKO, dilution 1:50) and SMCs (HHF35; DAKO, dilution 1:50) [20]. Control immunostainings were conducted without the primary antibodies. Detection of BrdU-positive cells was done according to manufacture's instructions. Morphometry and analysis of I/M ratio were performed using Olympus AX70 microscope and analysis software (Soft Imaging Systems, GmbH) [18]. Apoptosis was detected with ApopTag Kit according to the manufacturer's instructions (Intergen Company).

2.5. Reverse transcription-polymerase chain reaction

Total RNA was isolated from the tissue samples after homogenisation in Trizol reagent (Gibco BRL) and treated with RQ1 RNAse-free DNAse (Promega). Four μg of total RNA was reverse-transcribed using random hexamer primers (Promega) and M-Mulv Reverse Transcriptase (New England BioLabs). cDNA was amplified by PCR using DyNAzyme^{TM} II DNA Polymerase (Finnzymes) and primers specific for human Lp-PLA$_2$ sequence as follows: forward 5′-TGGAGCAACGGTTATTCAG-3′; reverse 5′-TGGTTGTGTTAATGTTGGTCC-3′. Reaction was subjected to 45 cycles: denaturing at 94 °C for 1 min, annealing at 62 °C for 1 min and extension at 72 °C for 1 min. Extension in the final cycle was 7 min. One microliter of cDNA was amplified by PCR using primers specific for LacZ as follows: forward 5′-TGAGGGGACGACGACAGTAT-3′, reverse 5′-TTGGAGGCCTAGGCTTTTGC-3′. Reaction was subjected to 40 cycles: denaturation at 95 °C for 45 s, annealing at 58 °C for 45 s and extension at 72 °C for 50 s. Nested PCR was then performed using 5 μl product of the first PCR reaction: forward 5′-GGTAGAAGACCCCAAGGACTTT-3′, reverse 5′-CGCCATTCGCCATTCAG-3′. Reaction was subjected to 40 cycles: denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min. Size of the product was 218 bp. Identity of the PCR products was assessed by size fractionation on ethidium bromide-stained agarose gels.

2.6. Statistical analyses

ANOVA followed by modified *t*-test was used to evaluate statistical significances. A value of $P < 0.05$ was considered statistically significant. Numerical values for each measurement are shown as mean ± S.E.M.

3. Results

3.1. Clinical chemistry and expression of Lp-PLA$_2$ and LacZ

Plasma Lp-PLA$_2$ activity was measured before (day 0) and 3, 7, 14 and 28 days after AdLp-PLA$_2$ or AdLacZ gene transfer (Fig. 1). Plasma Lp-PLA$_2$ activity showed a statistically significant increase in the Lp-PLA$_2$ group (48.2 ± 4.2) as compared to the LacZ group (33.6 ± 3.51, $p < 0.05$) at two weeks time point. Lipid levels (total cholesterol and triglycerides) and safety markers (ASAT and CRP) did not show any significant differences between the study groups (Fig. 1).

Lp-PLA$_2$ mRNA expression in aortas was detected two and four weeks after the gene transfer (Fig. 2). Adenovirus biodistribution was determined from liver, spleen, kidney, lung and aorta. As expected, some positive x-gal staining was seen in spleen, liver, lung and aorta two weeks after the gene transfer (Fig. 3) and at four weeks time point in aorta (Fig. 3), spleen and liver (data not shown).

3.2. Histological analysis

To determine the effect of Lp-PLA$_2$ gene transfer on neointima formation I/M ratio was measured from aortic samples. Histological analysis showed significantly ($p < 0.005$) reduced neointimal thickening in the Lp-PLA$_2$ group (0.25 ± 0.03) as compared to the LacZ (0.45 ± 0.05) controls at two weeks time point. At four weeks time point I/M tended to be lower in the Lp-PLA$_2$ group (0.34 ± 0.05) as compared to the LacZ group (0.53 ± 0.06) ($p = 0.057$) (Fig. 4). Examples of hematoxylin-eosin staining and immunostainings of the transduced arteries are shown in Fig. 5. Intimal thickening in all arteries was composed predominantly of SMCs.

RAM-11 staining showed no significant differences in the macrophage count between the groups (data not shown). Endothelial regrowth was analyzed by measuring the length of intact endothelium from histological sections. Intact endothelium (%) in the Lp-PLA$_2$ group at two weeks time point was 62.2 ± 9.2 and in the LacZ group 48.7 ± 13.2. At four weeks time point the values were 70.0 ± 8.4 in the Lp-PLA$_2$ group and 73.1 ± 8.7 in the LacZ group. No significant differences were found between the groups in the regrowth of endothelium. The percentage of proliferating cells was analyzed by BrdU labelling. No statistical differences were detected at two weeks time point, but the Lp-PLA$_2$ group (1.8 ± 0.3) tended to have a lower proliferation rate than the LacZ group (2.2 ± 0.6). At four weeks time point the difference between the Lp-PLA$_2$ group (0.9 ± 0.2) and the LacZ group (1.8 ± 0.4) was significant ($p < 0.05$) (Fig. 6). Apoptosis was higher in the LacZ control vessels than in the Lp-PLA$_2$ group at two weeks time point (Fig. 5), but the difference was not present at four weeks time point (data not shown).

4. Discussion

In the present study we have shown that adenovirus mediated Lp-PLA$_2$ gene transfer resulted in a significant reduction in neointima formation in balloon-denuded rabbit aorta. The protective effect of Lp-PLA$_2$ on restenosis is likely to be due to its ability to remove PAF and PAF-like oxidized phospholipids formed during and after arterial in-

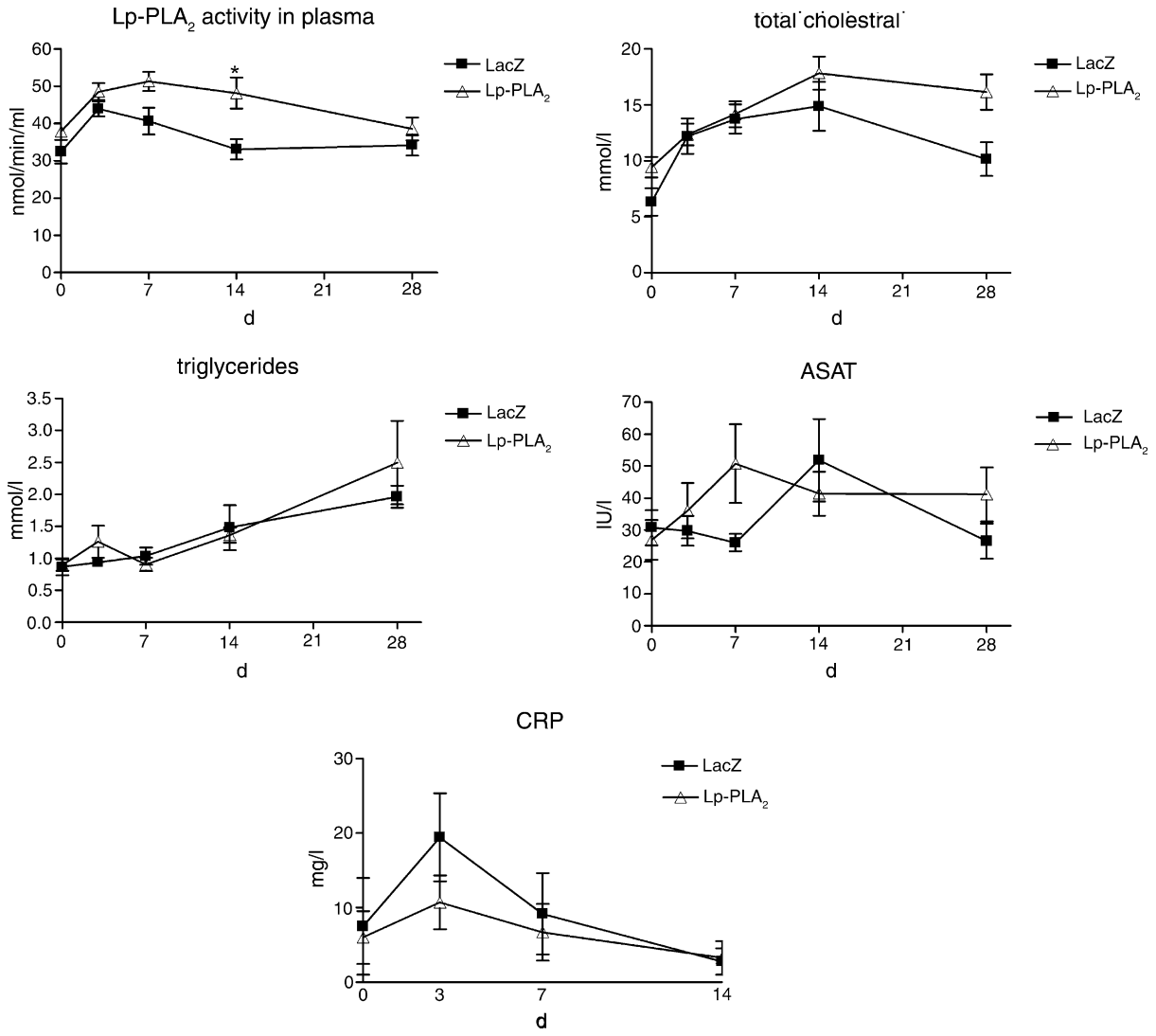

Fig. 1. Clinical chemistry analyses. Values (mean ± S.E.M.) of plasma Lp-PLA$_2$ activity, total cholesterol, triglycerides, aspartyl aminotransferase (ASAT) and C-reactive protein (CRP) after adenovirus-mediated LacZ or Lp-PLA$_2$ gene transfers. $^*P < 0.05$.

jury. It has been demonstrated that angioplasty is accompanied by platelet and leukocyte activation leading to increased PAF production and platelet-leukocyte adhesion [21,22]. PAF and PAF-like phospholipids are mitogens for SMCs and may thus contribute to the pathogenesis of restenosis [23,24]. In this study the injury in the vessel wall was made by balloon-denudation and the gene transfer was directed to the site of balloon-injury. Balloon denudation immediately increases O_2^- production [12] and also hypercholesterolemia contributes to this [25] suggesting a strong oxidant stress at the site of injury which can lead to formation of PAF-like lipids.

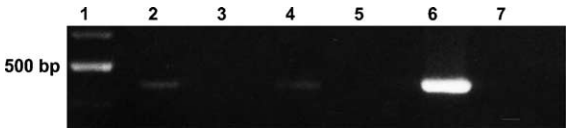

Fig. 2. Expression of human Lp-PLA$_2$ mRNA after adenoviral gene transfer in vivo. Lp-PLA$_2$ mRNA was detected by RT-PCR from aortas at two and four weeks time points. The product is 452 bp. Line 1: molecular weight marker; line 2: AdLp-PLA$_2$ at two weeks time point; line 3: AdLacZ at two weeks time point; line 4: AdLp-PLA$_2$ at four weeks time point; line 5: AdLacZ at four weeks time point; line 6: positive control (Lp-PLA$_2$ Adeno-X plasmid) and line 7: negative blank control.

First evidence of an inhibitory effect of Lp-PLA$_2$ on intimal hyperplasia was shown by Quarck et al., who demonstrated that systematically administered adenovirus-mediated gene transfer of human Lp-PLA$_2$ inhibited injury-induced neointima formation and resulted in reduced adhesion molecule expression and monocyte adhesion in apolipoprotein E-deficient mice [26]. In our rabbit model we used intravascular catheter-mediated gene transfer method in order to achieve local production of the treatment gene in the vessel wall, but with secreted gene product also systemic effects were achieved as seen in this study with elevated serum Lp-PLA$_2$ levels. Some Lp-PLA$_2$ adenovirus was also distributed into the liver and according to our pervious studies this leads

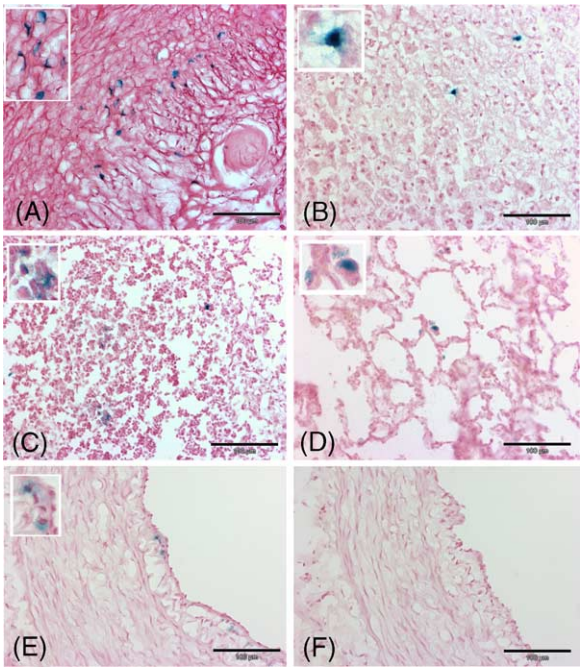

Fig. 3. X-gal staining to determine biodistribution of adenoviruses two (A–D) and four weeks (E) after LacZ gene transfer. (A) Aorta; (B) liver; (C) spleen; (D) lung; (E) aorta; (F) Lp-PLA$_2$ transduced aorta; two weeks time point. No staining was detected in heart and kidneys (data not shown). Bars = 100 μm.

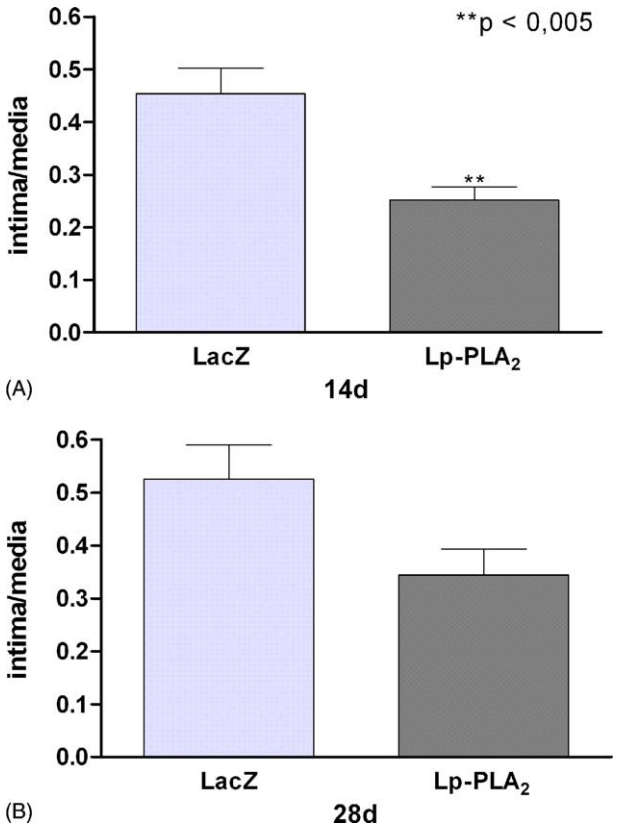

Fig. 4. Intima/media ratio (I/M) in the study groups after balloon denudation and gene transfer (mean ± S.E.M.). (a) I/M two weeks after the gene transfer. $^{*}P < 0.005$. (b) I/M four weeks after the gene transfer.

to the expression of Lp-PLA$_2$ in the liver and formation of LDL particles with increased Lp-PLA$_2$ activity which protects LDL against oxidative modification and degradation in macrophages [17]. A small portion of circulating enzyme activity is associated with HDL and there are several lines of evidence suggesting the antiatherogenic effects of Lp-PLA$_2$ bound to HDL [26–28]. However, the role of LDL-associated Lp-PLA$_2$ remains controversial and needs further investigation [29,30].

Endothelial dysfunction can result in the release of growth factors promoting vascular cell proliferation. Lp-PLA$_2$ gene transfer reduced apoptosis at two weeks time point. The exact role of apoptosis in restenosis in unknown, but at early stages after vascular injury apoptosis stimulates restenosis by provoking the wound-healing process. Apoptotic cells release cytokines and this could enhance the proliferative response after balloon injury [31]. There are many studies about the antiapoptotic effects of Lp-PLA$_2$-like activity [32,33] including the study by Chen et al, who showed that recombinant

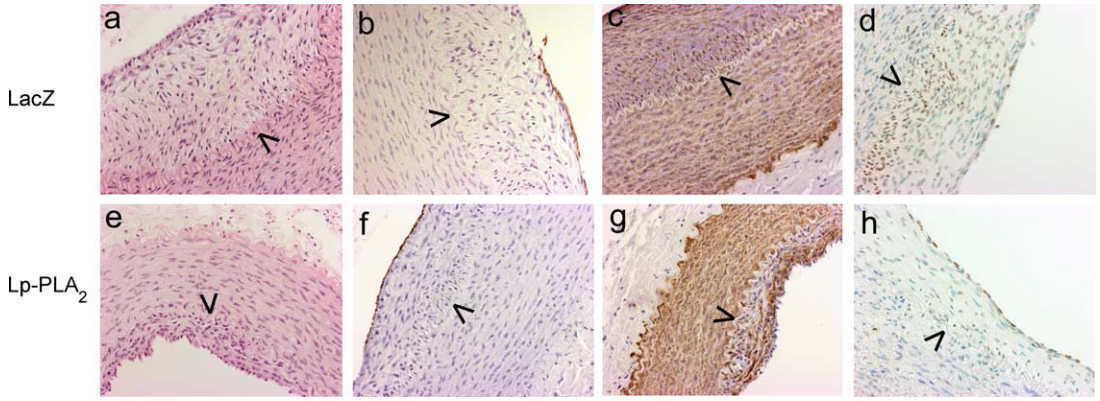

Fig. 5. Histological characterization of balloon-denuded aortas two weeks after gene transfer. (a–d) LacZ adenovirus transduced aortas. (a) HE-staining; (b) endothelium-specific immunostaining (CD-31); (c) α-actin immunostaining (HHF-35); (d) ApopTag staining. (e–h) Lp-PLA$_2$ adenovirus transduced aortas. (e) HE-staining; (f) endothelium-specific immunostainings (CD-31); (g) α-actin immunostaining (HHF-35); (h) ApopTag staining. Arrowheads denote internal elastic lamina. Magnification ×20.

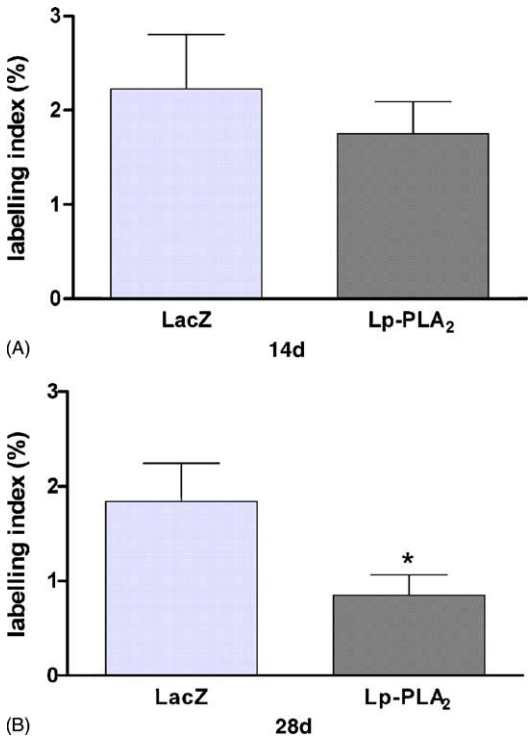

Fig. 6. Cell proliferation after balloon denudation and gene transfer. BrdU labelling showed lower proliferation rate in the AdLp-PLA$_2$ group as compared to the AdLacZ group. (a) Two weeks after the gene transfer. (b) Four weeks after the gene transfer. $^*P < 0.05$.

Lp-PLA$_2$, inhibited apoptosis induced by mildly oxidized LDL in cultured vascular endothelial cells and prevented calcium influx into neutrophils [34]. This anti-apoptotic effect of Lp-PLA$_2$ could be important in the inhibition of neointima formation, because it has been shown that early inhibition of apoptosis after balloon injury reduces neointimal hyperplasia [35]. In addition to reducing apoptosis, Lp-PLA$_2$ gene transfer lowered the proliferation rate which is in line with the observation that Lp-PLA$_2$ gene transfer led to the lower I/M ratios at both time points.

In conclusion, our results show that local cathether-mediated delivery of Lp-PLA$_2$ adenoviruses can reduce restenosis in rabbits and suggest that local administration of Lp-PLA$_2$ adenovirus could become a new approach for the prevention of restenosis after vascular manipulations.

Acknowledgements

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References

[1] Bittl JA. Advances in coronary angioplasty [published erratum appears in N Engl J Med 1997 Feb 27;336(9):670]. N Engl J Med 1996;335:1290–302.
[2] Ip JH, Fuster V, Badimon L, et al. Syndromes of accelerated atherosclerosis: role of vascular injury and smooth muscle cell proliferation. J Am Coll Cardiol 1990;15:1667–87.
[3] Libby P, Schwartz D, Brogi E, Tanaka H, Clinton SK. A cascade model for restenosis. A special case of atherosclerosis progression. Circulation 1992;86:III47–52.
[4] Marmur JD, Poon M, Rossikhina M, Taubman MB. Induction of PDGF-responsive genes in vascular smooth muscle Implications for the early response to vessel injury. Circulation 1992;86:III53–60.
[5] Le Breton H, Plow EF, Topol EJ. Role of platelets in restenosis after percutaneous coronary revascularization. J Am Coll Cardiol 1996;28:1643–51.
[6] Stahl GL, Terashita Z, Lefer AM. Role of platelet activating factor in propagation of cardiac damage during myocardial ischemia. J Pharmacol Exp Ther 1988;244:898–904.
[7] Stremler KE, Stafforini DM, Prescott SM, McIntyre TM. Human plasma platelet-activating factor acetylhydrolase. Oxidatively fragmented phospholipids as substrates. J Biol Chem 1991;266:11095–103.
[8] Neumann FJ, Ott I, Gawaz M, Puchner G, Schomig A. Neutrophil and platelet activation at balloon-injured coronary artery plaque in patients undergoing angioplasty. J Am Coll Cardiol 1996;27:819–24.
[9] Triggiani M, Schleimer RP, Warner JA, Chilton FH. Differential synthesis of 1-acyl-2-acetyl-sn-glycero-3-phosphocholine and platelet-activating factor by human inflammatory cells. J Immunol 1991;147:660–6.
[10] Stoll LL, Spector AA. Interaction of platelet-activating factor with endothelial and vascular smooth muscle cells in coculture. J Cell Physiol 1989;139:253–61.
[11] Zimmerman GA, McIntyre TM, Prescott SM, Stafforini DM. The platelet-activating factor signaling system and its regulators in syndromes of inflammation and thrombosis. Crit Care Med 2002;30:S294–301.
[12] Souza HP, Souza LC, Anastacio VM, et al. Vascular oxidant stress early after balloon injury: evidence for increased NAD(P)H oxidoreductase activity. Free Radic Biol Med 2000;28:1232–42.
[13] Tardif JC, Cote G, Lesperance J, et al. Probucol and multivitamins in the prevention of restenosis after coronary angioplasty. Multivitamins and Probucol Study Group. N Engl J Med 1997;337:365–72.
[14] Laukkanen MO, Kivela A, Rissanen T, et al. Adenovirus-mediated extracellular superoxide dismutase gene therapy reduces neointima formation in balloon-denuded rabbit aorta. Circulation 2002;106:1999–2003.
[15] Tew DG, Southan C, Rice SQJ, et al. Purification, properties, sequencing, and cloning of a lipoprotein-associated, serine-dependent phospholipase involved in the oxidative modification of low-density lipoproteins. Arteriosclerosis Thromb Vasc Biol 1996;16:591–9.
[16] Laitinen M, Mäkinen K, Manninen H, et al. Adenovirus-mediated gene transfer to lower limb artery of patients with chronic critical leg ischemia. Hum Gene Ther 1998;9:1481–6.
[17] Turunen P, Jalkanen J, Heikura T, et al. Adenovirus-mediated gene transfer of Lp-PLA2 reduces LDL degradation and foam cell formation in vitro. J Lipid Res 2004;45:1633–9.
[18] Hiltunen MO, Laitinen M, Turunen MP, et al. Intravascular adenovirus-mediated VEGF-C gene transfer reduces neointima formation in balloon-denuded rabbit aorta. Circulation 2000;102:2262–8.
[19] Laitinen M, Hartikainen J, Hiltunen MO, et al. Catheter-mediated vascular endothelial growth factor gene transfer to human coronary arteries after angioplasty. Hum Gene Ther 2000;11:263–70.

[20] Leppanen O, Rutanen J, Hiltunen MO, et al. Oral imatinib mesylate (STI571/gleevec) improves the efficacy of local intravascular vascular endothelial growth factor-C gene transfer in reducing neointimal growth in hypercholesterolemic rabbits. Circulation 2004;109:1140–6.
[21] Mickelson JK, Lakkis NM, Villarreal-Levy G, Hughes BJ, Smith CW. Leukocyte activation with platelet adhesion after coronary angioplasty: a mechanism for recurrent disease? J Am Coll Cardiol 1996;28:345–53.
[22] Neumann FJ, Ott I, Gawaz M, Puchner G, Schomig A. Neutrophil and platelet activation at balloon-injured coronary artery plaque in patients undergoing angioplasty. J Am Coll Cardiol 1996;27:819–24.
[23] Stoll LL, Spector AA. Interaction of platelet-activating factor with endothelial and vascular smooth muscle cells in coculture. J Cell Physiol 1989;139:253–61.
[24] Heery JM, Kozak M, Stafforini DM, et al. Oxidatively modified LDL contains phospholipids with platelet- activating factor-like activity and stimulates the growth of smooth muscle cells. J Clin Invest 1995;96:2322–30.
[25] Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. J Clin Invest 1993;91:2546–51.
[26] Quarck R, De Geest B, Stengel D, et al. Adenovirus-mediated gene transfer of human platelet-activating factor-acetylhydrolase prevents injury-induced neointima formation and reduces spontaneous atherosclerosis in apolipoprotein E-deficient mice. Circulation 2001;103:2495–500.
[27] Theilmeier G, De GB, Van Veldhoven PP, et al. HDL-associated PAF-AH reduces endothelial adhesiveness in apoE-/- mice. FASEB J 2000;14:2032–9.
[28] Eisaf M, Tselepis AD. Effect of hypolipidemic drugs on lipoprotein-associated platelet activating factor acetylhydrolase. Implication for atherosclerosis. Biochem Pharmacol 2003;66:2069–73.
[29] Chen CH. Platelet-activating factor acetylhydrolase: is it good or bad for you? Curr Opin Lipidol 2004;15:337–41.
[30] Caslake MJ, Packard CJ, Suckling KE, et al. Lipoprotein-associated phospholipase A(2), platelet-activating factor acetylhydrolase: a potential new risk factor for coronary artery disease. Atherosclerosis 2000;150:413–9.
[31] Walsh K, Smith RC, Kim HS. Vascular cell apoptosis in remodeling, restenosis, and plaque rupture. Circ Res 2000;87(3):184–8.
[32] Matsuzawa A, Hattori K, Aoki J, Arai H, Inoue K. Protection against oxidative stress-induced cell death by intracellular platelet-activating factor-acetylhydrolase II. J Biol Chem 1997;272:32315–20.
[33] Kuijpers TW, van den Berg JM, Tool AT, Roos D. The impact of platelet-activating factor (PAF)-like mediators on the functional activity of neutrophils: anti-inflammatory effects of human PAF-acetylhydrolase. Clin Exp Immunol 2001;123:412–20.
[34] Chen CH, Jiang T, Yang JH, et al. Low-density lipoprotein in hypercholesterolemic human plasma induces vascular endothelial cell apoptosis by inhibiting fibroblast growth factor 2 transcription. Circulation 2003;107:2102–8.
[35] Beohar N, Flaherty JD, Davidson CJ, et al. Antirestenotic effects of a locally delivered caspase inhibitor in a balloon injury model. Circulation 2003;6;109(1):108–13.

III

Effects of Vaccinia Virus Anti-Inflammatory Protein 35K and TIMP-1 Gene Transfers on Vein Graft Stenosis in Rabbits

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In Vivo, 19(3) in press (2005)

Effects of Vaccinia Virus Anti-Inflammatory Protein 35K and TIMP-1 Gene Transfers on Vein Graft Stenosis in Rabbits

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Abstract. *Vein graft stenosis is a common problem after bypass surgery. Vein grafts are ideal targets for gene therapy because transduction can be made ex vivo before grafting. Since chemokines and inflammatory factors are involved in vein graft thickening we tested a hypothesis that vaccinia virus anti-inflammatory protein 35K, which can sequester CC-chemokines, can reduce vein graft thickening in vivo.*

We used adenovirus mediated gene transfer (1×10^9 pfu/ml) of 35K and compared its effects on reducing stenosis in a rabbit jugular vein graft model with tissue inhibitor of metalloproteinase -1 (TIMP-1) and LacZ control gene. TIMP-1 was used in this study because it has been shown previously to inhibit vein graft stenosis in other model systems. Expression of transgenes in the transduced segments was confirmed by RT-PCR. Vein grafts were analyzed using immunohistological and morphometric methods at three day time point and at two weeks and four weeks time points.

It was found that the anti-inflammatory protein 35K was an efficient factor in reducing neointima formation at two weeks time point indicating that inflammatory factors play an important role in the vein graft stenosis. At four weeks time point 35K still showed a reduced accumulation of macrophages. TIMP-1 also tended to reduce neointimal thickening at two weeks time point as compared to lacZ.

It was found that 35K is an efficient factor in reducing neointima formation, macrophage accumulation and proliferation in rabbit vein grafts after adenoviral ex vivo gene transfer.

Abbreviations: TIMP-1, tissue inhibitor of metalloproteinase-1; SMC, smooth muscle cell; MMP, matrix metalloproteinase; ECM, extracellular matrix; CMV, cytomegalo virus; BrdU, 5-bromo-2-deoxyuridine; VCAM, vascular cell adhesion molecule; MCP-1, Monocyte chemotactic protein-1; KO, knock out.

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Key words: vein graft stenosis; gene therapy; adenovirus; inflammation

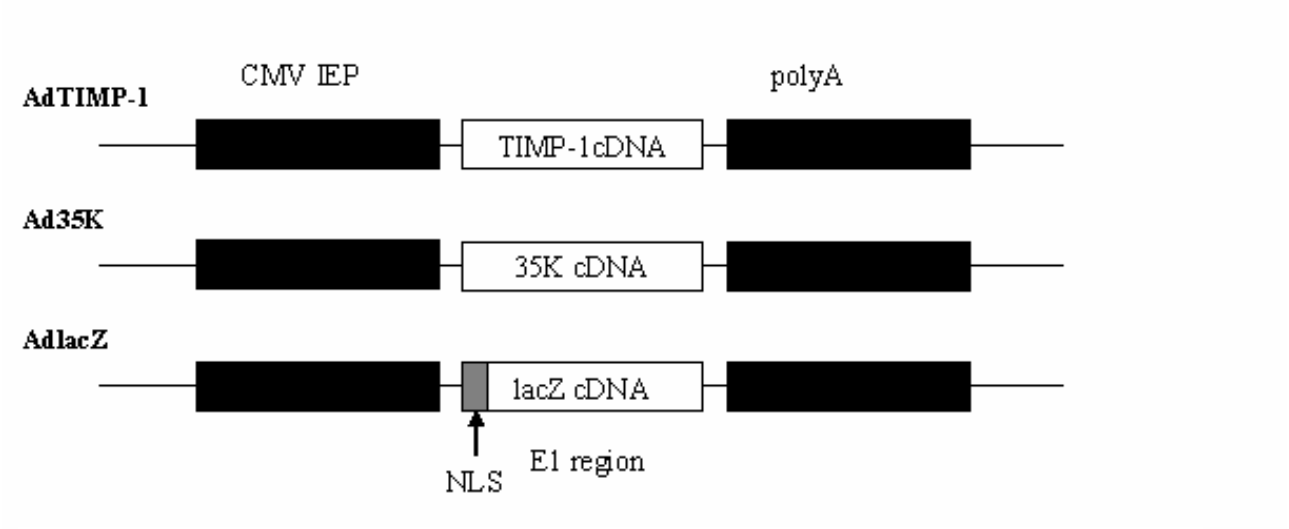

Figure 1. *Transgene structures in the adenoviral vectors. The same CMV promoters and poly A sequences were used for each transgene. Recombination resulted in the insertion of the expression cassette into the E1 gene region of the Ad5 genome. IEP: immediate early promoter; nls: nuclear location signal.*

Vein graft stenosis is a common problem after bypass operations leading to significant morbidity, mortality and cost. 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 (1). After that the neointimal growth gradually occludes the vein graft. The vein is always subjected to focal endothelial damages, no matter how subtle the surgical operation is. Intimal hyperplasia generates the foundation for the development of graft atheroma. Smooth muscle cell (SMC) proliferation is induced by cytokines and growth factors released from endothelial cells, platelets and macrophages (2). Anti-platelet therapy and lipid-lowering drugs have been used to prevent thrombotic occlusions and neointima formation, respectively, but still by ten years only 60% of the vein grafts are patent and only half of the patent vein grafts are free of significant stenosis (3) . Even though the use of arterial conduits has increased, only a small number of patients have had a full arterial revascularization. Thus, there is a great need to prolong the lifespan of the venous grafts.

Vein grafts are ideal targets for gene therapy because explanted veins can be transduced before grafting (4). The ideal vector should achieve efficient transduction and sustained gene expression with minimal toxicity. Adenoviruses are the most efficient viral vectors, but they suffer from cytotoxicity and harmful immune responses if too high titers are used (4). However, these problems can be reduced by local ex vivo gene transfer, which was used in the current study.

In this study we tested the treatment effects of vaccinia virus anti-inflammatory protein 35K and tissue inhibitor of metalloproteinase-1 (TIMP-1) which influence different pathological mechanisms in vein graft stenosis. 35K is a 35kDa vaccinia virus protein which binds with high affinity to chemokines of the CC-class (5). In vitro studies have shown that 35K inhibits chemokine signal transduction and cell migration (6). It may therefore be used to block CC-chemokine-induced monocyte/macrophage recruitment and several inflammatory factors associated with the progression of vein graft stenosis.

Matrix metalloproteinases (MMPs) are capable of degrading all components of the

extracellular matrix (ECM) and therefore they have been implicated in the progression of vascular diseases (7). Increased expression of MMP-2 and MMP-9 has been demonstrated in atherosclerotic plaques (8). Surgical injuries increase MMP-9 expression and MMP-2 activation in human saphenous veins (9). Since TIMP-1 is an endogenous inhibitor of MMP-2 and MMP-9 (8), we hypothesized that it should reduce neointima formation in the vein grafts. Furthermore, TIMP-1 gene transfer has been shown to prevent restenosis in a rat angioplasty model and in a human saphenous vein organ culture model (10;11). Also, targeted TIMP-1 gene transfer has reduced restenosis in a rabbit aortic restenosis model (12). Therefore, we also hypothesized that TIMP-1 could act as a positive control in our vein graft stenosis model.

In this study we tested whether anti-inflammatory effects of 35K and prevention of the degradation of ECM by TIMP-1 could reduce neointima formation. It was found that the anti-inflammatory protein 35K showed promising effects on reducing neointima formation and macrophage accumulation in the vein grafts.

Materials and Methods

Materials
All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co.

Adenoviruses
Replication-deficient E1-E3 deleted TIMP-1 (13;14), 35K (6) and lacZ (15) adenoviruses were produced in 293 cells as described (15). Helper-free viruses were produced 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. All viral lots were analyzed for the absence of microbiological contaminants, mycoplasma and lipopolysaccharide. The expression of all transgenes was driven by the CMV promoter and the lacZ adenovirus also had a nuclear location signal (figure 1).

Vein graft model
All studies were approved by Experimental Animal Committee of the University of Kuopio. Fifty New Zealand White male rabbits were randomly divided into TIMP-1, 35K or lacZ groups for two weeks and four weeks time points (five rabbits in each study group) and for two three day groups (35K and lacZ). Virus was used at the titer of 1.0×10^9 pfu /ml. Channon et al have shown that this dose is optimal for efficient transduction and gene expression in veins (16).

End to side anastomosis was made to the right common carotid artery using the right external jugular vein. The vein was first dissected free from the surrounding tissues and all branches were ligated. The proximal side of the vein was clamped and the bifurcation branches were ligated. A hole was made in the right bifurcation vein behind the ligation 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 injection of 500µl of the virus. The virus was kept in the vein for 10 min with a slight pressure. Virus was then removed, the vein was flushed with saline and detached just below the bifurcation. No virus was released to the systemic circulation. Carotid artery was dissected free form surrounding tissues 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. After anastomosis was finished the clip from the proximal end of the vein was removed. Clips from the carotid artery were then removed and blood flow was established. Four hours before sacrifice the rabbits were injected with BrdU (50 mg dissolved in 40% ethanol). Rabbits were sacrificed 3 days, two weeks and four weeks after the surgery and gene transfer.

RT-PCR

For the assessment of TIMP-1 mRNA expression, RT-PCR was performed from vein segments 2 and 4 weeks after the gene transfer. Total RNA was isolated using Trizol Reagent (Gibco-BRL) and 5 μg RNA was reverse-transcribed. 3 μl of cDNA was amplified by PCR using primers specific for TIMP-1 as follows: forward 5'-ACCCAACGACGGCCTTCTGCAATTC-3'; reverse 5'-GGCTATCTGGGACCGCAGGGACTGC-3'. Reaction was subjected to 40 cycles: denaturation at 95 ˚C for 1 min, annealing at 60 ˚C for 1 min and extension at 72˚C for 1 min. Size of the product was 500bp. Similarly, 3 μl of cDNA was amplified by PCR using primers specific for 35K as follows: forward 5'-ATCCTCATCCTCCTCCTCGT-3', reverse 5'-CTCAGACCTCCACCGATGAT-3'. Reaction was subjected to 35 cycles: denaturation at 94°C for 45 s, annealing at 55°C for 1 min and extension at 72 °C for 1 min. Size of the product was 243 bp. 1 μl of cDNA was amplified by PCR using primers specific for lacZ as follows: forward 5'-TGAGGGGACGACGACAGTAT-3', reverse 5'-TTGGAGGCCTAGGCTTTTGC-3'. Reaction was subjected to 40 cycles: denaturation at 95°C for 45 s, annealing at 58°C for 45 s and extension at 72 °C for 50 s. Nested PCR was then performed using 5 μl product of the first PCR reaction: forward 5'-GGTAGAAGACCCCAAGGACTTT-3', reverse 5'-CGCCATTCGCCATTCAG-3'. Reaction was subjected to 40 cycles: denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72 °C for 1 min. Size of the product was 218 bp. All RT-PCR tests were repeated twice. Identity of the PCR products was assessed by size fractionation on ethidium bromide-stained agarose gels.

Tissue samples and histology

After sacrifice the transduced segment was removed, flushed with saline and divided into six equal parts. The most proximal and the most distal parts of the vein were snap frozen in liquid nitrogen and stored at –70 °C. These parts were used for RT-PCR analyses. The next proximal and the next distal segments were immersion-fixed in 4% paraformaldehyde/phosphate buffered saline (pH 7.4) for 10 min, embedded in OCT-compound and stored at –70 °C. These parts were used for lacZ expression analyses. The two middle segments were immersion-fixed in 4% paraformaldehyde/15% sucrose (pH 7.4) for 4 h, rinsed in 15% sucrose (pH 7.4) overnight and embedded in paraffin. These parts were used for immunohistochemical stainigs and for morphometry which were analyzed from four randomly selected sections per graft for each different immunostaining and morphometrical measurements.

Immunohistochemical stainings were performed for the middle parts of the vein for the detection of macrophages (RAM-11; DAKO, dilution 1:50), endothelium (CD31; DAKO, dilution 1:50), BrdU (DAKO, dilution 1:100) and apoptosis (Intergen Company) (17). Macrophage count was obtained by counting immunopositive cells in the intimal and medial layers per slide (figure 5). Endothelial cell coverage was obtained by measuring the length of the immunopositive intact endothelial cell layer and the total lumen circumference. Intact endothelium was expressed as a ratio of the measured endothelium and the lumen circumference (%). Apoptosis was detected by positive immunohistochemical staining and expressed as an apoptosis score per slide (18). Control immunostainings were done without the primary antibodies and with class and species matched irrelevant primary antibodies. Morphometry and analysis of the intima/media (I/M) ratio were performed by measuring the areas of intimal-layer and medial-layer and calculating the ratio (19). Olympus AX70 microscope and analySIS software were used for the analyses (Soft imaging systems, GmbH) (19).

Statistical analysis
Statistical analysis was performed using Chi-square test or ANOVA, followed by modified Student's t-test. Results were considered significant at $p<0.05$ value.

Results

Expression of transduced genes
Gene transfer studies in vein grafts were performed with Ad35K, AdTIMP-1 and AdLacZ at a viral titer of 1.0×10^9 pfu/ml. Expression of all transduced genes was detected in the vein grafts from the most proximal and the most distal segments with RT-PCR (figure 2).

Histological analysis
To determine the effects of gene transfers on neointima formation I/M ratios were measured by using the areas of intimal and medial layers (figure 3). No differences were found in I/M ratios three days after the gene transfer. At three day time point I/M ratios were (0.19±0.04) in Ad35K group and (0.24±0.03) in AdlacZ group. Significantly ($p< 0.05$) reduced neointimal hyperplasia was found in Ad35K group (0.24±0.04) at two weeks time point as compared to AdLacZ group (0.42±0.05), whereas I/M in AdTIMP-1 (0.30±0.1) group did not reach statistical significance. At four weeks time point 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). Representative examples of the vein graft histology are shown in figure 4.

Macrophage accumulation is part of the inflammatory response in the vein grafts. At three day time point no macrophages were present, which is probably due to the short period of time after the surgery. At two weeks time point there were no differences in the macrophage count. However, we noticed that macrophage accumulation was significantly lower ($p<0.05$) in Ad35K group at four weeks time point as compared to AdlacZ group (figure 5).

Surgical manipulation and rapid changes in blood pressure always cause some damages to the endothelium. Endothelial coverage in the vein grafts after the gene transfer was analyzed by measuring the % length of the intact endothelium from the lumen circumference from histological sections immunostained for CD31. No differences were found three days after the operation. At two weeks time point the endothelial coverage tended to be higher in Ad35K group (95%±4.9) but the difference was not statistically significant. At four weeks time point no statistically significant differences were present (figure 6).

The percentage of proliferating cells was measured by BrdU labelling. At three day time point no SMC proliferation was detected which could be due to the short perioid after the operation. There were no statistically significant differences between the groups at two weeks time point, but at four weeks time point AdTIMP-1 group (0.42%±0.11) showed a significant difference ($p<0.05$) as compared to AdlacZ group (0.70%±0.3) (figure 7). On the other hand, Ad35K and AdTIMP-1 groups showed significantly reduced apoptosis scores ($p<0.05$, Chi-square test) at four weeks time point as compared to AdlacZ group (table 1). No apoptosis was detected at three day time point.

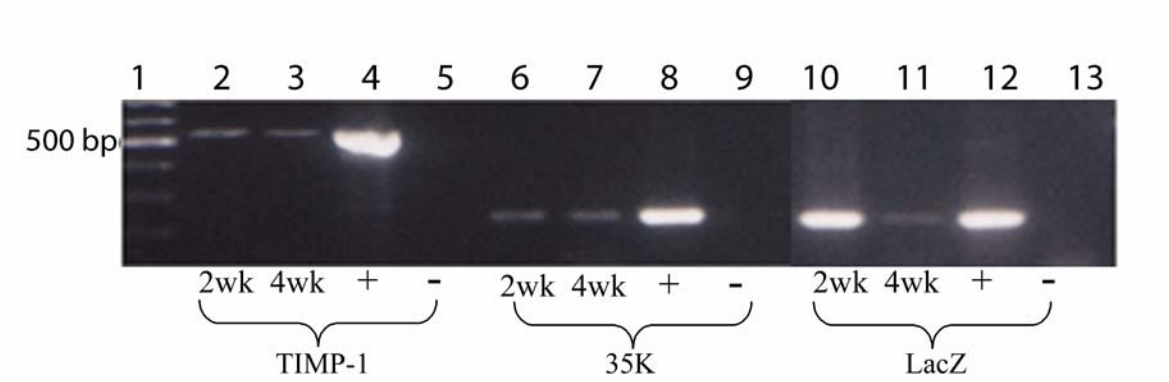

Figure 2. *Expression of TIMP-1, 35K and LacZ in transduced vein grafts. mRNAs were detected by RT-PCR at two and four weeks time points. Line 1: molecular weight marker; Lines 2 and 3: TIMP-1 expression at two and four weeks time points; Line 4: AdTIMP-1 control for TIMP-1; Line 5: uninfected control for TIMP-1; Lines 6 and 7: 35K expression at two and four weeks time points; Line 8: Ad35K control for 35K; Line 9: uninfected control for 35K; Lines 10 and 11: LacZ expression at two and four weeks time points; Line 12: AdLacZ control for LacZ; Line 13: uninfected control for LacZ.*

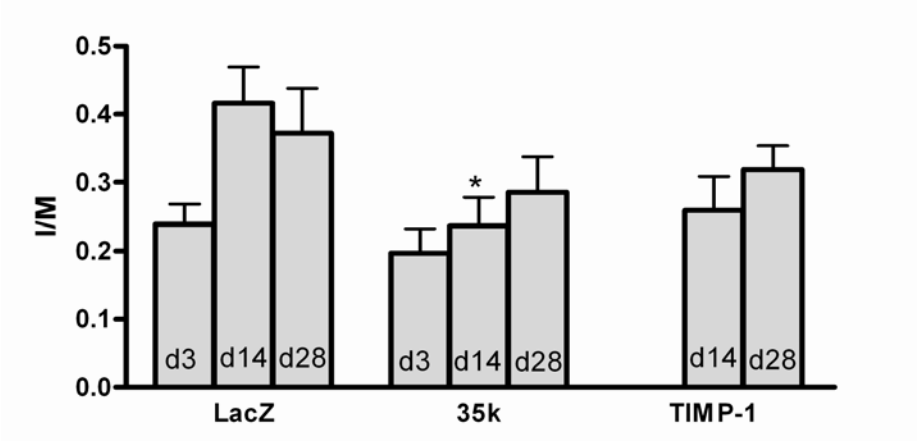

Figure 3. *Neointima formation in vein grafts after the gene transfer (mean ± SEM). The I/M ratios were measured by using the areas of intima and media in hematoxylin-eosin stained histological sections. Four randomly selected sections were analyzed per each graft. Five male rabbits were operated in each treatment group. *P<0.05 in comparison to the AdlacZ group at the same time point.*

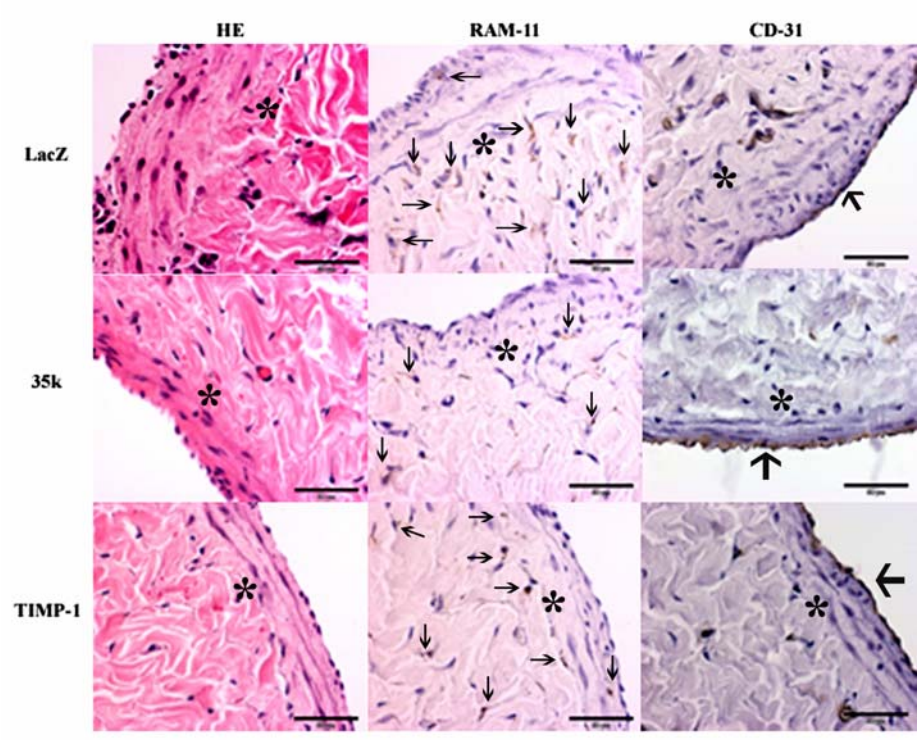

Figure 4. *Histological analysis of vein grafts two weeks after the gene transfer. HE, macrophage (RAM-11, dilution 1:50) and endothelium (CD31, dilution 1:50) stainings. Treatment groups are indicated as lacZ, 35K and TIMP-1 in the figure. Arrows point at positive macrophages in RAM-11 stainings and endothelium in CD31 stainings. Five male rabbits were operated in each treatment group. Magnification x 40. Bars 50 µm. Star * indicates the border of intima-media.*

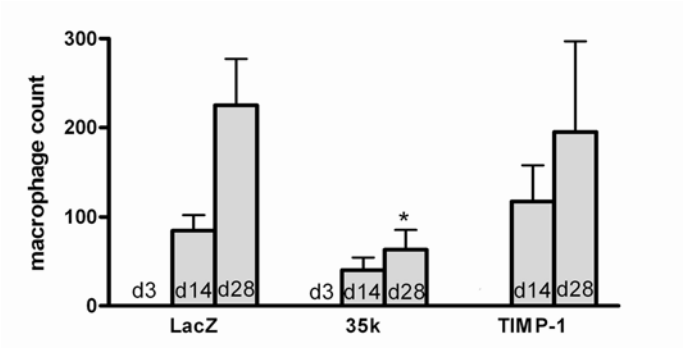

Figure 5. *Macrophace infiltration in the vein grafts after the gene transfer (mean ± SEM). Macrophages were counted as positive cells in RAM-11 immunostaining. y-axis indicates the number of macrophages per slide. Four randomly selected sections were analyzed per each graft. Five male rabbits were operated in each treatment group. *P<0.05 in comparison to the AdlacZ group at the same time point.*

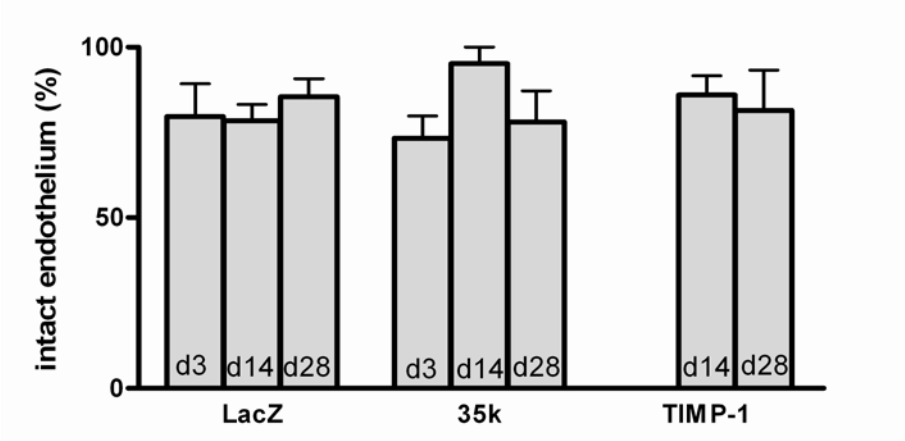

Figure 6. *Endothelial damage was evaluated by measuring intact endothelium in the vein grafts as detected by CD31 immunostaining (mean ± SEM). y-axis indicates the percentage of intact endothelium from the total lumen circumference per slide. Four randomly selected sections were analyzed per each graft. Five male rabbits were operated in each treatment group.*

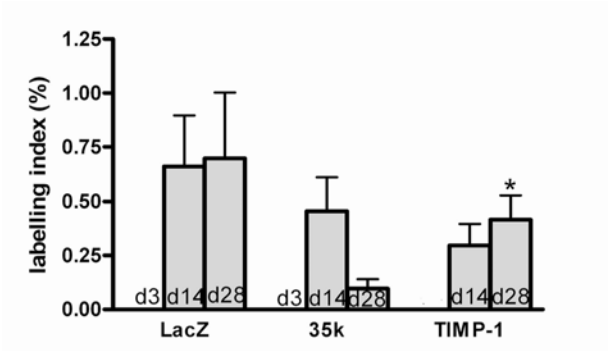

Figure 7. *Cell proliferation index in the vein grafts after the gene transfer (mean ± SEM). Cell proliferation was measured by using BrdU labeling and counting the positive cells per slide. Four randomly selected sections were analyzed per each graft. Five male rabbits were operated in each treatment group. *P<0.05 in comparison to the AdlacZ group at the same time point.*

A) **2 wk** positive staining	LacZ	35k	TIMP-1
1-2 (low)	6	4	4
3-4 (high)	7	3	10
chi-test p-value		0.2198	0.3445
B) **4 wk** positive staining	LacZ	35k	TIMP-1
1-2 (low)	0	7	9
3-4 (high)	7	2	4
chi-test p-value		0.0019 **	0.003 **

Table 1. *Apoptosis as detected by positive immunohistochemical staining (low staining scores: (1-2), high staining scores (3-4)) Chi-square test. **P<0.005 in comparison to the AdlacZ group at the same time point. Four randomly selected sections were analyzed per each graft. Five male rabbits were operated in each treatment group.*

Discussion

Although a number of studies have addressed the potential use of gene transfer for the treatment of arterial restenosis (4), very little is known about gene transfer to vein grafts: 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 oligonucleotide gene therapy in a rabbit model (20). Chen et al used adenoviral gene transfer of soluble VCAM in porcine interposition vein grafts which reduced neointimal thickening (21). It has also been shown that sdi-1, a mediator of tumor suppressor p53 action, inhibited neointima formation in rabbit vein grafts (22). 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 (23). However, TIMP-3 was found to inhibit neointima formation in a porcine vein graft model (24). Also, adenoviral gene transfer of eNOS has been reported to reduce neointima formation (25). According to our knowledge, this is the first study comparing different potential treatment genes using adenovirus vector in the rabbit jugular vein graft model.

Our hypothesis was that gene transfers with Ad35K and AdTIMP-1 during the vein graft operation might be effective in reducing neointimal thickening. Since gene transfers were made *ex vivo* no virus was exposed to systemic circulation. Selection of the therapeutic genes was based on the pathophysiological mechanisms of vein graft stenosis. Vaccinia virus anti-inflammatory protein 35K was selected because it is known to bind efficiently to CC-chemokines like MCP-1 (6). We hypothetisized that 35K would inhibit inflammatory responses and macrophage accumulation, since intimal hyperplasia can be considered as vascular wound healing response after vein-grafting where monocytes and macrophages have a central role (26). It has been shown that MCP-1 gene is upregulated early after surgery (27), suggesting a central role of CC-chemokines in intimal hyperplasia. TIMP-1 has been previously shown to reduce neointima formation in arterial restenosis models and in a human saphenous vein organ culture model (28). We assumed that TIMP-1 could also have an effect in the rabbit vein graft model. AdTIMP-1 group also helped to evaluate the

magnitude of the potential effect obtained by Ad35K which has not been previously used for the treatment of arterial restenosis or vein graft thickening. However, recently it has been shown, that adenovirus-mediated gene transfer of 35K reduced atherosclerosis and inhibited CC-CK-induced macrophage recruitment in atherosclerotic ApoE KO mice (29).

It was found that the anti-inflammatory protein 35K significantly reduced neointima formation at two weeks time point as compared to the control. TIMP-1 also showed a tendency towards reduced neointima formation but the difference did not reach statistical significance. The efficacy of TIMP-3 gene transfer has been shown in a porcine vein graft model (30). On the other hand, no previous studies have been reported on TIMP-1 gene transfer in the rabbit vein graft model. At four weeks time point all treatment groups had reached the I/M ratio seen in the control group. Previous studies have shown that adenoviral transgene expression decreases after two weeks time point (19), and therefore the result was not unexpected. Thus, only short term effects were achieved, which may be due to the transient nature of the adenoviral gene transfer effect.

It was also found that macrophage accumulation was significantly decreased at four weeks time point in Ad35K group as compared to AdlacZ group. Also, Ad35K group showed a tendency towards a decreased SMC proliferation index at four weeks time point. This may be due to the anti-inflammatory properties of 35K which seems to delay the overall inflammation response in the vessel wall. Therefore, it's not unexpected that changes in the proliferation index and in the accumulation of macrophage can still be seen at four weeks time point. 35K can block the CC- chemokines which could lead to the reduced macrophage accumulation and SMC proliferation. However, AdTIMP-1 group also had a significantly decreased proliferation rate at four weeks time point. It looks like the neointima formation is influenced at least by two processes: inflammation and cell proliferation. Using 35K gene transfer inflammation and cell proliferation can be reduced whereas with TIMP-1 gene transfer only cell proliferation can be influenced. Decreased apoptosis was evident at four weeks time point both in 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 injury. Therefore, the decreased apoptosis rate could reflect the additive effects of decreased inflammation and lower rate of SMC proliferation and macrophage accumulation (31).

We conclude that the anti-inflammatory effects of 35K may be useful for the inhibition of vein graft stenosis. Also, it's ability to decrease macrophage accumulation and SMC proliferation could be beneficial in the treatment of vein graft stenosis.

Acknowledgements

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References

1. Motwani,J.G. and Topol,E.J. (1998) Aortocoronary saphenous vein graft disease: Pathogenesis, predisposition, and prevention, *Circulation 97*, 916-931.

2. Ku,D.D., Caulfield,J.B., and Kirklin,J.K. (1991) Endothelium-dependent responses in long-term human coronary artery bypass grafts, *Circulation 83*, 402-411.

3. Fitzgibbon,G.M., Kafka,H.P., Leach,A.J., Keon,W.J., Hooper,G.D., and Burton,J.R. (1996) Coronary bypass graft fate and patient outcome: angiographic follow-up of 5,065 grafts related to survival and reoperation in 1,388 patients during 25 years, *J. Am. Coll. Cardiol. 28*, 616-626.

4. Ylä-Herttuala,S. and Martin,J.F. (2000) Cardiovascular gene therapy, *Lancet 355*, 213-222.

5. Seet,B.T., Singh,R., Paavola,C., Lau,E.K., Handel,T.M., and McFadden,G. (2001) Molecular determinants for CC-chemokine recognition by a poxvirus CC-chemokine inhibitor, *Proc. Natl. Acad. Sci. U. S. A 98*, 9008-9013.

6. Bursill,C.A., Cai,S., Channon,K.M., and Greaves,D.R. (2003) Adenoviral-mediated delivery of a viral chemokine binding protein blocks CC-chemokine activity in vitro and in vivo, *Immunobiology 207*, 187-196.

7. Dollery,C.M., McEwan,J.R., and Henney,A.M. (1995) Matrix metalloproteinases and cardiovascular disease, *Circ. Res. 77*, 863-868.

8. George,S.J. (1998) Tissue inhibitors of metalloproteinases and metalloproteinases in atherosclerosis, *Curr Opin Lipidol 9*, 413-423.

9. Zorina,S.G. and Jaikirshan,J.K. (2002) Matrix metalloproteinases in Vascular remodeling and Atherogenesis: The Good, the Bad and the Ugly, *Circ Res 90*, 251-262.

10. George,S.J., Johnson,J.L., Angelini,G.D., Newby,A.C., and Baker,A.H. (1998) Adenovirus-mediated gene transfer of the human TIMP-1 gene inhibits smooth muscle cell migration and neointimal formation in human saphenous vein, *Hum Gene Ther 9*, 867-877.

11. Dollery,C.M., Humphries,S.E., McClelland,A., Latchman,D.S., and McEwan,J.R. (1999) Expression of tissue inhibitor of matrix metalloproteinases 1 by use of an adenoviral vector inhibits smooth muscle cell migration and reduces neointimal hyperplasia in the rat model of vascular balloon injury, *Circulation 99*, 3199-3205.

12. Turunen,M.P., Puhakka,H.L., Koponen,J.K., Hiltunen,M.O., Rutanen,J., Leppanen,O., Turunen,A.M., Narvanen,A., Newby,A.C., Baker,A.H., and Yla-Herttuala,S. (2002) Peptide-retargeted adenovirus encoding a tissue inhibitor of metalloproteinase-1 decreases restenosis after intravascular gene transfer, *Mol. Ther. 6*, 306-312.

13. Baker,A.H., Zaltsman,A.B., George,S.J., and Newby,A.C. (1998) Divergent effects of tissue inhibitor of metalloproteinase-1, -2, or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation, and death in vitro. TIMP-3 promotes apoptosis, *J. Clin. Invest. 101*, 1478-1487.

14. Baker,A.H., Wilkinson,G.W., Hembry,R.M., Murphy,G., and Newby,A.C. (1996) Development of recombinant adenoviruses that drive

high level expression of the human metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 and -2 genes: characterization of their infection into rabbit smooth muscle cells and human MCF-7 adenocarcinoma cells, *Matrix Biol. 15*, 383-395.

15. Laitinen,M., Mäkinen,K., Manninen,H., Matsi,P., Kossila,M., Agrawal,R.S., Pakkanen,T., Luoma,J.S., Viita,H., Hartikainen,J., Alhava,E., Laakso,M., and Ylä-Herttuala,S. (1998) Adenovirus-mediated gene transfer to lower limb artery of patients with chronic critical leg ischemia, *Hum Gene Ther 9*, 1481-1486.

16. Channon,K.M., Fulton,G.J., Gray,J.L., Annex,B.H., Shetty,G.A., Blazing,M.A., Peters,K.G., Hagen,P.O., and George,S.E. (1997) Efficient adenoviral gene transfer to early venous bypass grafts: comparison with native vessels, *Cardiovasc Res 35*, 505-513.

17. Leppanen,O., Rutanen,J., Hiltunen,M.O., Rissanen,T.T., Turunen,M.P., Sjoblom,T., Bruggen,J., Backstrom,G., Carlsson,M., Buchdunger,E., Bergqvist,D., Alitalo,K., Heldin,C.H., Ostman,A., and Yla-Herttuala,S. (2004) Oral imatinib mesylate (STI571/gleevec) improves the efficacy of local intravascular vascular endothelial growth factor-C gene transfer in reducing neointimal growth in hypercholesterolemic rabbits, *Circulation 109*, 1140-1146.

18. Pulkkanen,K.J., Laukkanen,J.M., Fuxe,J., Kettunen,M.I., Rehn,M., Kannasto,J.M., Parkkinen,J.J., Kauppinen,R.A., Pettersson,R.F., and Yla-Herttuala,S. (2002) The combination of HSV-tk and endostatin gene therapy eradicates orthotopic human renal cell carcinomas in nude mice, *Cancer Gene Ther. 9*, 908-916.

19. Hiltunen,M.O., Laitinen,M., Turunen,M.P., Jeltsch,M., Hartikainen,J., Rissanen,T.T., Laukkanen,J., Niemi,M., Kossila,M., Hakkinen,T.P., Kivela,A., Enholm,B., Mansukoski,H., Turunen,A.M., Alitalo,K., and Yla-Herttuala,S. (2000) Intravascular adenovirus-mediated VEGF-C gene transfer reduces neointima formation in balloon-denuded rabbit aorta, *Circulation 102*, 2262-2268.

20. Ehsan,A., Mann,M.J., Dell'Acqua,G., and Dzau,V.J. (2001) Long-term stabilization of vein graft wall architecture and prolonged resistance to experimental atherosclerosis after E2F decoy oligonucleotide gene therapy, *J. Thorac. Cardiovasc. Surg. 121*, 714-722.

21. Chen,S.J., Wilson,J.M., and Muller,D.W. (1994) Adenovirus-mediated gene transfer of soluble vascular cell adhesion molecule to porcine interposition vein grafts, *Circulation 89*, 1922-1928.

22. Bai,H., Morishita,R., Kida,I., Yamakawa,T., Zhang,W., Aoki,M., Matsushita,H., Noda,A., Nagai,R., Kaneda,Y., Higaki,J., Ogihara,T., Sawa,Y., and Matsuda,H. (1998) Inhibition of intimal hyperplasia after vein grafting by in vivo transfer of human senescent cell-derived inhibitor-1 gene, *Gene Ther 5*, 761-769.

23. George,S.J., Baker,A.H., Angelini,G.D., and Newby,A.C. (1998) Gene transfer of tissue inhibitor of metalloproteinase-2 inhibits metalloproteinase activity and neointima formation in human saphenous veins, *Gene Ther 5*, 1552-1560.

24. George,S.J., Lloyd,C.T., Angelini,G.D., Newby,A.C., and Baker,A.H. (2000) Inhibition of late vein graft neointima formation in human and porcine models

by adenovirus-mediated overexpression of tissue inhibitor of metalloproteinase-3, *Circulation 101*, 296-304.

25. Cable,D.G., Obrien,T., Schaff,H.V., and Pompili,V.J. (1997) Recombinant endothelial nitric oxide synthase transduced human saphenous veins: gene therapy to augment nitric oxide production in bypass conduits, *Circulation 96*, Suppl.):173-178.

26. Eslami,M.H., Gangadharan,S.P., Belkin,M., Donaldson,M.C., Whittemore,A.D., and Conte,M.S. (2001) Monocyte adhesion to human vein grafts: a marker for occult intraoperative injury?, *J. Vasc. Surg. 34*, 923-929.

27. Stark,V.K., Hoch,J.R., Warner,T.F., and Hullett,D.A. (1997) Monocyte chemotactic protein-1 expression is associated with the development of vein graft intimal hyperplasia, *Arterioscler. Thromb. Vasc. Biol. 17*, 1614-1621.

28. Turunen,M.P., Puhakka,H.L., Koponen,J.K., Hiltunen,M.O., Rutanen,J., Leppanen,O., Turunen,A.M., Narvanen,A., Newby,A.C., Baker,A.H., and Yla-Herttuala,S. (2002) Peptide-retargeted adenovirus encoding a tissue inhibitor of metalloproteinase-1 decreases restenosis after intravascular gene transfer, *Mol. Ther. 6*, 306-312.

29. Bursill,C.A., Choudhury,R.P., Ali,Z., Greaves,D.R., and Channon,K.M. (2004) Broad-spectrum CC-chemokine blockade by gene transfer inhibits macrophage recruitment and atherosclerotic plaque formation in apolipoprotein E-knockout mice, *Circulation 110*, 2460-2466.

30. George,S.J., Lloyd,C.T., Angelini,G.D., Newby,A.C., and Baker,A.H. (2000) Inhibition of late vein graft neointima formation in human and porcine models
by adenovirus-mediated overexpression of tissue inhibitor of metalloproteinase-3, *Circulation 101*, 296-304.

31. Beohar,N., Flaherty,J.D., Davidson,C.J., Maynard,R.C., Robbins,J.D., Shah,A.P., Choi,J.W., MacDonald,L.A., Jorgensen,J.P., Pinto,J.V., Chandra,S., Klaus,H.M., Wang,N.C., Harris,K.R., Decker,R., and Bonow,R.O. (2004) Antirestenotic effects of a locally delivered caspase inhibitor in a balloon injury model, *Circulation 109*, 108-113.

IV

Combination Gene Therapy of EC-SOD with 35k or TIMP-1 in the Treatment of Vein Graft Stenosis in Rabbits

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Submitted for publication

Combination Gene Therapy of EC-SOD with 35k or TIMP-1 in the Treatment of Vein Graft Stenosis in Rabbits

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Abstract

Introduction: Vein graft stenosis is a common problem after bypass operation. Neointima formation in vein graft stenosis is affected by oxidative stress, acute inflammatory response and proliferation. Gene therapy offers a novel treatment strategy for vein graft stenosis because gene transfer can be done ex vivo during the graft operation.

Methods: In this study we used adenovirus-mediated ex vivo gene transfer of extracellular superoxide dismutase (EC-SOD) alone or in combination with tissue inhibitor of metalloproteinase-1 (TIMP-1) or vaccinia virus anti-inflammatory protein 35k to prevent vein graft stenosis in a jugular vein graft model of normocholesterolemic New Zealand White rabbits. Vein grafts were analyzed 14 and 28 days after the gene transfer using histological methods.

Results: It was found that at two weeks time point the combinations of EC-SOD+35k and EC-SOD + TIMP-1 gene transfers were most effective in decreasing neointima formation. A long term effect was seen in the EC-SOD+TIMP-1 combination group which also showed decreased proliferation index. The combination of anti-inflammatory proteins (EC-SOD+35k) was most effective in reducing macrophage accumulation which was still significant at four weeks time point. ***Conclusion:*** Oxidative, inflammatory and proliferation processes are important for neointima formation in vein graft stenosis. In a rabbit model of vein graft disease combination gene therapy of anti-inflammatory and anti-proliferative proteins was effective in decreasing neointima formation. This may be because two different treatment genes can more efficiently affect pathological events at early stages more efficiently than gene transfer of just one gene.

Introduction

Coronary artery bypass grafting (CAGB) is a common treatment method for coronary heart disease. However, in 50% of the patients vein graft stenosis causes recurring symptoms within ten years of operation leading to reoperation[1]. Obviously, secondary prevention, including treatment of hypertension and hyperlipidemia and quitting smoking after CABG, is far from optimal and need to be improved. However, there is still a clear need to develop new methods for the prevention of vein graft stenosis and prolonging the lifespan of vein grafts.

The pathogenesis of vein graft stenosis is a complex process. There are at least three factors which contribute to the formation of vein graft stenosis. Thrombotic occlusion is the biggest problem after the operation [2] and therefore anti-platelet therapy has been used to prevent thrombotic occlusions. Gradually, neointimal formation develops and contributes to the occlusion of the vein graft. The neointimal formation requires migration and proliferation of smooth muscle cells (SMCs) which are induced by cytokines and growth factors released from endothelial cells, platelets and macrophages. Intimal hyperplasia generates the foundation for the development of graft atheroma. The vein is always subjected to focal endothelial damages, no matter how subtle the surgical operation is. It has been implicated that biomechanical forces, such as wall tension and shear stress, have been shown to be critical factors of vascular remodeling and intimal hyperplasia. Blood flow and shear stress on the endothelium have been identified as important regulators of both the biochemical and morphological changes that occur during early graft remodelling [3].

Vein grafts are ideal targets for gene therapy because explanted veins can be transduced before grafting [4]. The ideal vector should achieve efficient transduction and sustained gene expression with minimal toxicity. Adenoviruses are the most efficient viral vectors, but they suffer from cytotoxicity and harmful immune responses if too high titers are used [4]. However, local ex vivo gene transfer reduces these problems.

Very little data are available about combination gene therapy in the treatment of vascular diseases. Leppänen et al have shown that combination of intravascular VEGF-C gene transfer and treatment with PDGF receptor kinase inhibitor STI571 leads to long-term reduction of neointima formation in balloon-denuded rabbit aorta [5] .

Vaccinia-virus anti-inflammatory protein 35k binds with high affinity to CC-class chemokines [6]. It may therefore be used to block CC-chemokine-induced monocyte/macrophage recruitment and several inflammatory factors associated with the progression of vein graft stenosis. Recently Bursill et al showed that a single intravenous injection of a recombinant adenovirus encoding the broad-spectrum CC-inhibitor 35k can reduce atherosclerosis by inhibiting CC-CK-induced macrophage recruitment in atherosclerotic ApoE knock-out mice [7]. EC-SOD has been shown to reduce O_2^- -mediated macromolecular and cellular damage, therefore suggesting that EC-SOD gene transfer may be used to attenuate tissue damage caused by oxygen-derived free radicals [8, 9]. Laukkanen et al showed that local catheter-mediated delivery of EC-SOD adenoviruses can reduce aortic restenosis in rabbits [10]. Matrix remodelling is considered to be fundamental to the initiation of neointimal hyperplasia in vein-grafts allowing SMC migration which is dependent on activation of MMPs [11]. Expression of MMP-2 and MMP-9 is increased in vein graft segments contributing to intimal thickening [12]. Activity of MMPs is regulated by the interaction with TIMPs and TIMP-1 is found to act as an endogenous inhibitor of MMP-2 and MMP-9 [13]. It has been shown that adenovirus-mediated TIMP-1 gene transfer reduces MMP activity in vein graft segments and decreases neointimal formation [14].

In our previous studies we have established a rabbit vein graft model and studied the effects of a single treatment gene on the vein graft stenosis. We showed that vaccinia-virus anti-inflammatory protein 35k reduces vein graft stenosis. However, the effect of the single gene treatment was short-lived and at four weeks time point the study groups had similar intima/media (I/M) ratios as compared to the control group (Puhakka et al. In Vivo 2005, in press). In this study we hypothesized that combination gene therapy should reduce neointima formation and prolong the treatment effect in the rabbit vein graft model. Our study is the first one to compare multiple gene effects on the vein graft model in rabbits. It was found that the combination of EC-SOD and TIMP-1 gene therapy was more effective than the single EC-SOD gene therapy in decreasing proliferation and inflammatory effects in the vein grafts.

Methods

Materials

All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co.

Adenoviruses

Replication-deficient E1-E3 deleted TIMP-1 [15, 16], 35k [6], EC-SOD and lacZ [17]adenoviruses were produced in 293 cells as described [17]. Helper-free viruses were produced 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. All viral lots were analyzed for the absence of microbiological contaminants, mycoplasma and lipopolysaccharide. The expression of all transgenes was driven by the CMV promoter and the lacZ adenovirus also had a nuclear targeted signal.

Vein-graft model

All studies were approved by Experimental Animal Committee of the University of Kuopio. Seventy New Zealand White rabbits were randomly divided into AdEC-SOD, Ad(EC-SOD+35k), Ad(EC-SOD+TIMP-1), or AdlacZ groups for two weeks and four weeks time points (five rabbits in each study group). All viruses were used at the titer of 1.0×10^9 pfu /ml. Channon et al have shown that this dose is optimal for efficient transduction and gene expression in veins [18].

End to side anastomosis was made to the right common carotid artery using the right external jugular vein. The vein was first dissected free from the surrounding tissues and all branches were ligated. The proximal side of the vein was clamped and the bifurcation branches were ligated. A hole was made in the right bifurcation vein right behind the ligation 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 injection of 500µl of the virus. The virus was kept in the vein for 10 min with a slight pressure. Virus was then removed and the vein was flushed with saline. Carotid artery was dissected free form surrounding tissues 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. After anastomosis was finished the clip from the proximal end of the vein was removed. Clips from the carotid artery were removed and blood flow was established. Four hours before sacrifice the rabbits were injected with BrdU (50 mg dissolved in 40% ethanol). Rabbits were sacrificed two weeks and four weeks after the surgery and gene transfer.

RT-PCR

For the assessment of transgene mRNA expression, RT-PCR was performed from vein segments two and four weeks after the gene transfer. Total RNA was isolated using Trizol Reagent (Gibco-BRL) and 5 μg RNA was reverse-transcribed. 3 μl of cDNA was amplified by PCR using primers specific for transgene. TIMP-1 primers were as follows: forward 5'-ACCCAACGACGGCCTTCTGCAATTC-3'; reverse 5'-GGCTATCTGGGACCGCAGGGACTGC-3'. Reaction was subjected to 40 cycles: denaturation at 95 ˚C for 1 min, annealing at 60 ˚C for 1 min and extension at 72˚C for 1 min. Size of the product was 500bp. Similarly, 3 μl of cDNA was amplified by PCR using primers specific for 35k as follows: forward 5'-ATCCTCATCCTCCTCCTCGT-3', reverse 5'-CTCAGACCTCCACCGATGAT-3'. Reaction was subjected to 35 cycles: denaturation at 94°C for 45 s, annealing at 55°C for 1 min and extension at 72 °C for 1 min. Size of the product was 243 bp. Specific primers for EC-SOD were: forward 5'-TGATGTTGGGCGACCG-3', reverse 5'-GGATGTTGCAAGTG ACCAGGC-3' and for the second, nested PCR: forward 5'-GTGAGCGCCTGCCAGATCTC-3' and reverse 5'- GGATGTTGCAAGTG ACCAGGC-3'. Annealing temperature was 60 ºC in both stages and reactions were subjected to 30 cycles. 1 μl of cDNA was amplified by PCR using primers specific for lacZ as follows: forward 5'-TGAGGGGACGACGACAGTAT-3', reverse 5'-TTGGAGGCCTAGGCTTTTGC-3'. Reaction was subjected to 40 cycles: denaturation at 95°C for 45 s, annealing at 58°C for 45 s and extension at 72 °C for 50 s. Nested PCR was then performed using 5 μl product of the first PCR reaction: forward 5'-GGTAGAAGACCCCAAGGACTTT-3', reverse 5'-CGCCATTCGCCATTCAG-3'. Reaction was subjected to 40 cycles: denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72 °C for 1 min. Size of the product was 218 bp. All RT-PCR tests were repeated twice. Identity of the PCR products was assessed by size fractionation on ethidium bromide-stained agarose gels.

Tissue samples and histology

After sacrifice the transduced segment was removed, flushed with saline and divided into six equal parts. Also, for biodistribution analyses samples from the lung, kidney, liver and spleen were collected and divided into two parts. The most proximal and the most distal parts of the vein graft and one part of the organs were snap frozen in liquid nitrogen and stored at –70 °C. The next proximal and the next distal segments of the veins were immersion-fixed in 4% paraformaldehyde/15% sucrose (pH 7.4) for 4 h, rinsed in 15% sucrose (pH 7.4) overnight and embedded in paraffin, and the two middle segments and the other half of the organ samples were immersion-fixed in 4% paraformaldehyde/phosphate buffered saline (pH 7.4) for 10 min, embedded in OCT-compound and stored at –70 °C.

In situ superoxide anion production was determined by the dehydroethidium (DHE) staining method. In the presence of superoxide anion, DHE is converted to a fluorescent molecule ethidium. Briefly, fresh-frozen sections (15 µm) of veins were incubated with DHE (Molecular Probes) for 5 min, rinsed, mounted, and analyzed using a fluorescent microscope. Only bright fluorescing cells were considered positive. The number of total nuclei was counted after DAPI staining and results were expressed as a ratio of DHE/DAPI.

Immunohistochemical stainings were performed for the detection of macrophages (RAM-11; DAKO, dilution 1:50), endothelium (CD31; DAKO, dilution 1:50), BrdU (DAKO, dilution 1:100) and apoptosis (Intergen Company). Macrophage count was obtained by counting immunopositive cells in the intima and media layers per slide. Endothelial cell coverage was obtained by measuring the immunopositive intact endothelial cell layer as percentage of the total lumen circumference. Apoptosis was evaluated by positive immunohistochemical scores. Control immunostainings were done without the primary antibodies and with class and species matched irrelevant primary antibodies. Morphometry and analysis of the intima/media (I/M) ratio were performed using Olympus AX70 microscope and analySIS software (Soft imaging systems, GmbH) [19].

Analysis of EC-SOD activity

Plasma EC-SOD activity was measured at time points d0, d7, d14 and d28 according to manufacturer's instructions by SOD Assay Kit-WST (Dojindo Molecular Technologies, Japan) which utilizes water-soluble tetrazolium salt, WST-1 (2-(4- Iodophenyl)-3-(4-nitrophenyl)-5-(2,4 disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a

water-soluble formazan dye upon reduction by the superoxide anion. Since the absorbance at 440 nm is proportional to the amount of superoxide anion, the SOD activity as an inhibitory activity can be quantified by measuring the decrease in the colour development.

Statistical analysis

Statistical analysis was performed using Chi-square test or ANOVA, followed by modified Student's t-test. Results were considered significant at $p<0.05$ value.

Results

Expression of transduced genes

Gene transfer studies in vein grafts were performed with AdEC-SOD, Ad(EC-SOD+35k), Ad(EC-SOD+TIMP-1) and AdLacZ at a viral titer of 1.0×10^9 pfu/ml/virus. Expression of all transduced genes was detected in vein grafts with RT-PCR at two weeks and four weeks time point (figure 1.)

Histological analysis

To determine the effects of gene transfers on neointima formation I/M ratios were measured from vein grafts (Figure 2a). Significantly ($p< 0.05$) reduced I/M ratio was found in Ad(EC-SOD+35k) (0.19±0.10) and Ad(EC-SOD+TIMP-1) (0.18±0.02) at two weeks time point as compared to AdLacZ group (0.42±.17), whereas I/M ratio in AdEC-SOD (0.28±0.08) group did not reach statistical significance. At four weeks time point I/M ratio was significantly ($p<0.05$) reduced in Ad(EC-SOD+TIMP-1) group (0.16±0.03) as compared to control AdlacZ (0.34±0.15) group. Decreased I/M levels were noticed in the AdEC-SOD (0.20±0.15) group but the differences did not reach statistical significance. Ad(EC-SOD+35k) (0.35±0.21) showed a similar I/M level as control lacZ group. These data suggest that combination gene therapy with EC-SOD and TIMP-1 may prolong the treatment effect.

Cytokines cause macrophage accumulation which is an essential part of the inflammatory response. At two weeks time point Ad(EC-SOD+35k) showed a significantly ($P<0.05$) reduced macrophage count as compared to the control AdlacZ group. At four weeks time point Ad(EC-SOD+35k) still significantly reduced number of the macrophages as compared to the control AdlacZ group. Similarly, AdEC-SOD showed a decreased macrophage count at four weeks time point (figure 2b).

Endothelial coverage in vein grafts after the gene transfer was analyzed by measuring the length of the intact endothelium from histological sections. At two weeks time point AdEC-SOD (95.4 % ±5.0) group showed a significantly ($p<0.05$) higher endothelial coverage as compared to the control AdlacZ group. At four weeks time point no statistically significant differences were present (Figure 2c).

The numbers of proliferating cells was measured by BrdU labelling (cells/mm2) (Figure 2d). At two weeks time point the Ad(EC-SOD+TIMP-1) (8.50±6.6) group showed significantly ($p<0.05$) decreased proliferating levels as compared to the control AdlacZ group (19.7±9.1). At four weeks time point Ad(EC-SOD+35k) group (1.90±2.0) showed a significantly decreased proliferation index as compared to the control group. Apoptosis was detected by positive staining scores (1-2: low, 3-4: high frequency of

apoptosis). Apoptosis was decreased significantly in AdEC-SOD group in two and four weeks time points compared to the AdLacZ controls. In addition, Ad(EC-SOD+35k) gene transfer showed a significant reduction in apoptosis (table 1).

X-gal stainings (Figure 3) showed transduction in the endothelial and intimal cells of the vein grafts at two weeks time point. In the biodistribution studies at two weeks time point positive lacZ cells were found only in the spleen. This could be due to the local adenoviral exposure in the vein graft adventitia. Stainings from lung, kidney and liver did not show any positive staining.

Superoxide anion production in the vessel wall was determined by DHE assay two weeks and four weeks after the gene transfer. Numbers of the total nuclei were counted after DAPI staining and the values were calculated as a ratio of DHE/DAPI. Analyses revealed a significantly ($P<0.05$) reduced DHE staining in the AdEC-SOD (0.33±0.1) and Ad(EC-SOD+TIMP-1) (0.38±0.01) groups compared to the AdLacZ group (0.43±0.04) (figure 4). Examples of DHE stainings are shown in figure 4.

Analysis of EC-SOD activity

Plasma EC-SOD activity was measured before (day 0) and 7, 14 and 28 days after the gene transfer (figure 5). Plasma SOD activity showed a statistically significant ($P<0.05$) increase in Ad(EC-SOD+35k) (82%±0.35 inhibition) and AdEC-SOD (81%±0.63) inhibition) and AdEC-SOD (81%±0.63) groups as compared to the AdLacZ group (75%±1.38) at seven days time point and Ad(EC-SOD+35k) (86%±9.35) group also at two weeks time point as compared to the AdLacZ group (76%±2.23).

Discussion

Failure of vein bypass grafts is a common clinical problem that causes significant morbidity and mortality. Pathogenesis of vein graft stenosis is a complex process. Cell proliferation and inflammatory processes are involved in the gradual vein occlusion. Studies show that 60% of grafts occlude within 10 years time after the CABG operation. Therefore new strategies to prolong the lifespan of the vein grafts are needed. Vein grafts provide a unique opportunity for gene therapy, since the target tissue is available for local ex vivo gene application prior to graft implantation.

Previous studies have been successful mainly with E2F decoy by cell cycle gene blockade therapy [20]. Mann et al have already progressed to clinical studies with E2F [21]. Since the pathogenesis of vein graft stenosis in not yet fully understood, further studies are needed to get better understanding of the mechanisms leading to stenosis. In this study we used combination gene therapy to study which mechanisms might be crucial to decrease intimal hyperplasia. Previously we showed that vaccinia virus anti-inflammatory protein 35k efficiently reduced neointima formation in vein grafts (Puhakka et al., In Vivo 2005, in press). Also, TIMPs are known to have anti-proliferative effects in various stenosis models [14, 22, 23]. Anti-oxidative and anti-inflammatory EC-SOD has shown great promise in decreasing restenosis in aortic restenosis. Using these gene combinations, we assumed that better results might be achieved. To our knowledge there are no previous combination gene therapy studies for the treatment of vein graft stenosis and EC-SOD gene transfer has never before examined in vein grafts.

At two weeks time point the combination of Ad(EC-SOD +35k) and Ad(EC-SOD+TIMP-1) showed reduced levels of intimal hyperplasia. At four weeks time point the effect was seen in Ad(EC-SOD+TIMP-1) group suggesting that the combination of anti-inflammatory, anti-oxidative and anti-proliferative proteins may be beneficial in the treatment of vein graft stenosis. The combination of Ad(EC-SOD+35k) was the most efficient in reducing macrophage accumulation which was still significant at four weeks time point. EC-SOD gene transfer reduced also the production of superoxide anion at two weeks time point. These findings indicate that oxidative stress plays an important role in the pathogenesis of vein graft stenosis. All vein grafts undergo a period of ischemia followed by reperfusion which leads to the local generation of superoxide within the wall, triggering secondary inflammatory cascades and direct cytotoxicity to resident ECs and SMCs [24] . Gene therapy with anti-oxidative EC-SOD

and anti-inflammatory 35k might be effective by scavenging oxygen species and inflammatory chemokines and protecting cells in vascular wall. Similar results have been shown in previous restenosis studies in arteries, where gene transfer of an antioxidant has shown the potential to prevent restenosis and atherosclerosis progression [10, 25]. Probucol is the only antioxidant so far which has been shown to reduce restenosis in clinical trials [26 27]. Countacting oxidative stress may limit lipoprotein oxidation, endothelial dysfunction, and vascular inflammation.

Surgical manipulations and the rapid changes in the blood pressure in the graft after implantation cause damages to the endothelium. Intact endothelial coverage was measured form the grafts. In general, endothelial levels were similar in every group, however, at two weeks time point the AdEC-SOD group had a significantly higher endothelial coverage level as compared to the control lacZ group. This could be due to the EC-SODs anti-oxidative property. The sudden change in the graft blood pressure initiates oxidative processes which EC-SOD may inhibit. Actually, Yet et al have shown that endogenously induced production of heme oxygenase-1 (HO-1), which is an anti-oxidative protein, plays a protective role in vein graft stenosis. In the response to hemodynamic stress the HO-1-mice had much greater neointima formation than the wild type mice [28].

Our study is the first one to compare combination gene therapy effects on the vein graft thickening in the rabbit model. We found that the combination gene therapy of EC-SOD with TIMP-1 is effective in decreasing neointima formation still at four weeks time point. This may be because two different treatment genes can more efficiently affect different pathological events at early stages and degenerative changes more efficiently than just the transfer of one gene. Since pathological events in vein graft stenosis happen early after operation and it is important to slow down the pathological cascade as soon as possible. When using proper gene combinations, this can be probably achieved more efficiently than with single gene therapy.

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Reference List

(1) Campeau L, Enjalbert M, Lesperance J, Vaislic C, Grondin CM, Bourassa MG. Atherosclerosis and late closure of aortocoronary saphenous vein grafts: sequential angiographic studies at 2 weeks, 1 year, 5 to 7 years, and 10 to 12 years after surgery. *Circulation* 1983 September;68(3 Pt 2):II1-II7.

(2) Motwani JG, Topol EJ. Aortocoronary saphenous vein graft disease: Pathogenesis, predisposition, and prevention. *Circulation* 1998 March 10;97(9):916-31.

(3) Zarins CK, Giddens DP, Bharadvaj BK, Sottiurai VS, Mabon RF, Glagov S. Carotid bifurcation atherosclerosis. Quantitative correlation of plaque localization with flow velocity profiles and wall shear stress. *Circ Res* 1983 October;53(4):502-14.

(4) Ylä-Herttuala S, Martin JF. Cardiovascular gene therapy. *Lancet* 2000 January 15;355:213-22.

(5) Leppanen O, Rutanen J, Hiltunen MO et al. Oral imatinib mesylate (STI571/gleevec) improves the efficacy of local intravascular vascular endothelial growth factor-C gene transfer in reducing neointimal growth in hypercholesterolemic rabbits. *Circulation* 2004 March 9;109(9):1140-6.

(6) Bursill CA, Cai S, Channon KM, Greaves DR. Adenoviral-mediated delivery of a viral chemokine binding protein blocks CC-chemokine activity in vitro and in vivo. *Immunobiology* 2003;207(3):187-96.

(7) Bursill CA, Choudhury RP, Ali Z, Greaves DR, Channon KM. Broad-spectrum CC-chemokine blockade by gene transfer inhibits macrophage recruitment and atherosclerotic plaque formation in apolipoprotein E-knockout mice. *Circulation* 2004 October 19;110(16):2460-6.

(8) Strålin P, Karlsson K, Johansson BO, Marklund SL. The interstitium of the human arterial wall contains very large amounts of extracellular superoxide dismutase. *Arterioscler Thromb Vasc Biol* 1995;15(11):2032-6.

(9) Luoma JS, Strålin P, Marklund SL, Hiltunen TP, Särkioja T, Ylä-Herttuala S. Expression of extracellular SOD and iNOS in macrophages and smooth muscle cells in human and rabbit atherosclerotic lesions - Colocalization with epitopes characteristic of oxidized LDL and peroxynitrite-modified proteins. *Arterioscler Thromb Vasc Biol* 1998 February;18(2):157-67.

(10) Laukkanen MO, Kivela A, Rissanen T et al. Adenovirus-mediated extracellular superoxide dismutase gene therapy reduces neointima formation in balloon-denuded rabbit aorta. *Circulation* 2002 October 8;106(15):1999-2003.

(11) Zorina SG, Jaikirshan JK. Matrix metalloproteinases in Vascular remodeling and Atherogenesis: The Good, the Bad and the Ugly. *Circ Res* 2002;90:251-62.

(12) George SJ, Zaltsman AB, Newby AC. Surgigal preparative injury and neointima formation increase MMP-9 expression and MMP-2 activation in human saphenous vein. *Cardiovasc Res* 1997;33:447-59.

(13) George SJ. Tissue inhibitors of metalloproteinases and metalloproteinases in atherosclerosis. *Curr Opin Lipidol* 1998 October;9(5):413-23.

(14) George SJ, Johnson JL, Angelini GD, Newby AC, Baker AH. Adenovirus-mediated gene transfer of the human TIMP-1 gene inhibits smooth muscle cell migration and neointimal formation in human saphenous vein. *Hum Gene Ther* 1998 April 10;9(6):867-77.

(15) Baker AH, Zaltsman AB, George SJ, Newby AC. Divergent effects of tissue inhibitor of metalloproteinase-1, -2, or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation, and death in vitro. TIMP-3 promotes apoptosis. *J Clin Invest* 1998 March 15;101(6):1478-87.

(16) Baker AH, Wilkinson GW, Hembry RM, Murphy G, Newby AC. Development of recombinant adenoviruses that drive high level expression of the human metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 and -2 genes: characterization of their infection into rabbit smooth muscle cells and human MCF-7 adenocarcinoma cells. *Matrix Biol* 1996 December;15(6):383-95.

(17) Laitinen M, Mäkinen K, Manninen H et al. Adenovirus-mediated gene transfer to lower limb artery of patients with chronic critical leg ischemia. *Hum Gene Ther* 1998 July 1;9(10):1481-6.

(18) Channon KM, Fulton GJ, Gray JL et al. Efficient adenoviral gene transfer to early venous bypass grafts: comparison with native vessels. *Cardiovasc Res* 1997 September;35(3):505-13.

(19) Hiltunen MO, Laitinen M, Turunen MP et al. Intravascular adenovirus-mediated VEGF-C gene transfer reduces neointima formation in balloon-denuded rabbit aorta. *Circulation* 2000 October 31;102(18):2262-8.

(20) Mann MJ. E2F decoy oligonucleotide for genetic engineering of vascular bypass grafts. *Antisense Nucleic Acid Drug Dev* 1998 April;8(2):171-6.

(21) Mann MJ, Whittemore AD, Donaldson MC et al. Ex-vivo gene therapy of human vascular bypass grafts with E2F decoy: the PREVENT single-centre, randomised, controlled trial. *Lancet* 1999 October 30;354(9189):1493-8.

(22) George SJ, Baker AH, Angelini GD, Newby AC. Gene transfer of tissue inhibitor of metalloproteinase-2 inhibits metalloproteinase activity and neointima formation in human saphenous veins. *Gene Ther* 1998 November;5(11):1552-60.

(23) George SJ, Lloyd CT, Angelini GD, Newby AC, Baker AH. Inhibition of late vein graft neointima formation in human and porcine models by adenovirus-mediated overexpression of tissue inhibitor of metalloproteinase-3. *Circulation* 2000 January 25;101(3):296-304.

(24) Shi Y, Patel S, Davenpeck KL et al. Oxidative stress and lipid retention in vascular grafts: comparison between venous and arterial conduits. *Circulation* 2001 May 15;103(19):2408-13.

(25) Azevedo LC, Pedro MA, Souza LC et al. Oxidative stress as a signaling mechanism of the vascular response to injury: the redox hypothesis of restenosis. *Cardiovasc Res* 2000 August 18;47(3):436-45.

(26) Cote G, Tardif JC, Lesperance J et al. Effects of probucol on vascular remodeling after coronary angioplasty. Multivitamins and Protocol Study Group. *Circulation* 1999 January 5;99(1):30-5.

(27) Tardif JC, Cote G, Lesperance J et al. Impact of residual plaque burden after balloon angioplasty in the MultiVitamins and Probucol (MVP) trial. *Can J Cardiol* 2001 January;17(1):49-55.

(28) Yet SF, Layne MD, Liu X et al. Absence of heme oxygenase-1 exacerbates atherosclerotic lesion formation and vascular remodeling. *FASEB J* 2003 September;17(12):1759-61.

Figures and Figure legends

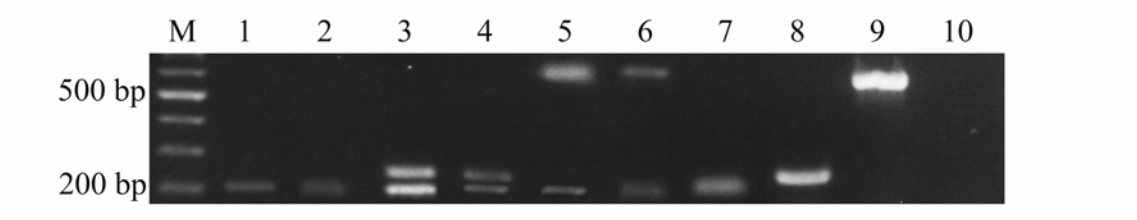

Figure 1. Expression of AdEC-SOD, Ad(EC-SOD+35k) and Ad(EC-SOD+TIMP-1) in transduced vein grafts. mRNAs were detected by RT-PCR at two and four weeks time points. M: molecular weight marker; line 1: AdEC-SOD 2 wk; line 2: AdEC-SOD 4 wk; line 3: Ad(EC-SOD+35k) 2 wk; line 4: Ad(EC-SOD+35k) 4 wk; line 5: Ad(EC-SOD+TIMP-1) 2 wk; line 6: Ad(EC-SOD+TIMP-1) 4 wk; line 7: AdEC-SOD positive control (218 bp); line 8: Ad35k positive control (243 bp); line 9: AdTIMP-1 positive control (500 bp); line 10: negative control (untransduced vein) for all transgenes.

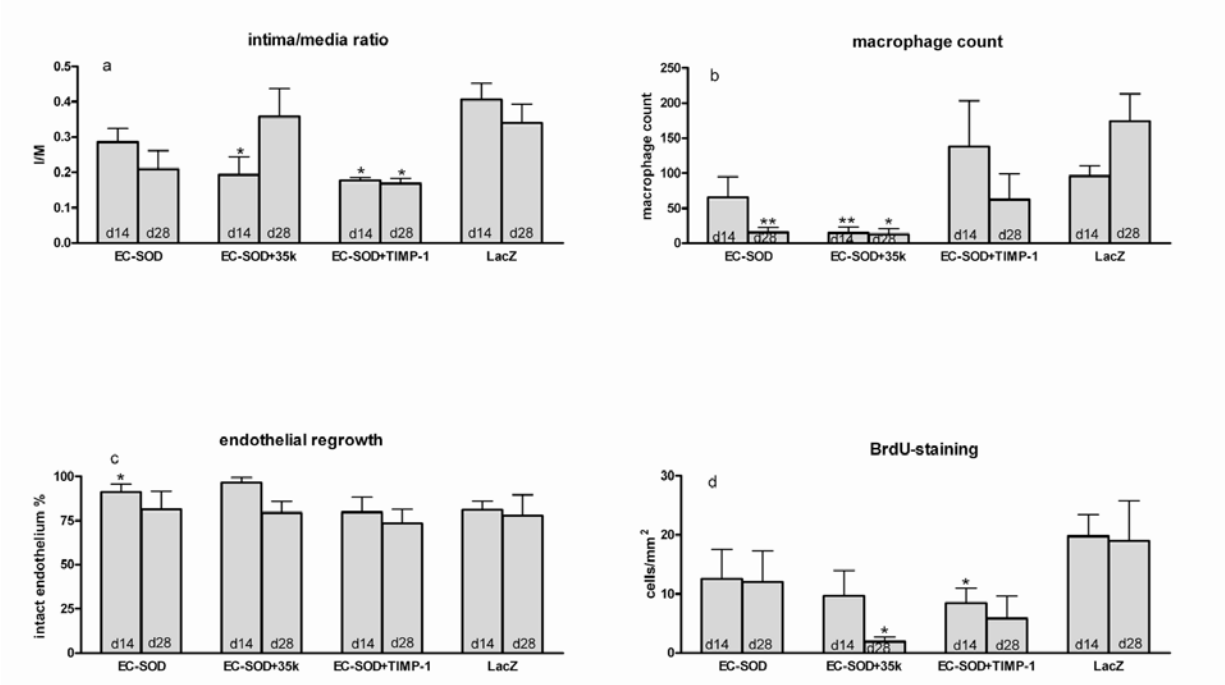

Figure 2. Intima/media ratios (a), macrophage count (b), endothelial regrowth (c), proliferation and BrdU-staining (d) in vein grafts two and four weeks after the gene transfer (mean ± SEM). *P<0.05, **P<0.01.

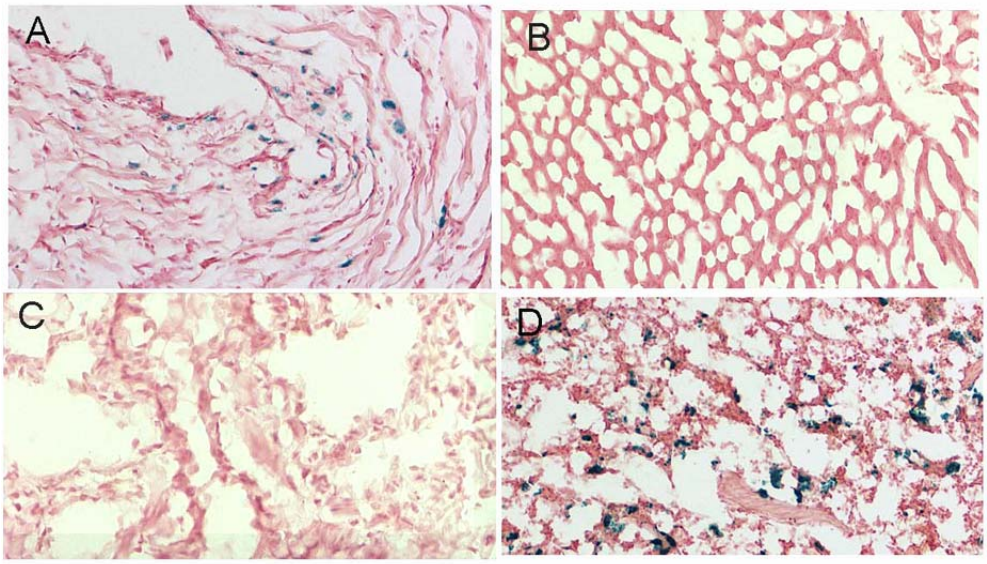

Figure 3. X-gal staining to determine gene transfer efficiency and biodistribution of adenoviruses two weeks after LacZ gene transfer. A: vein; B: liver; C: lung; D: spleen. Magnification x 200.

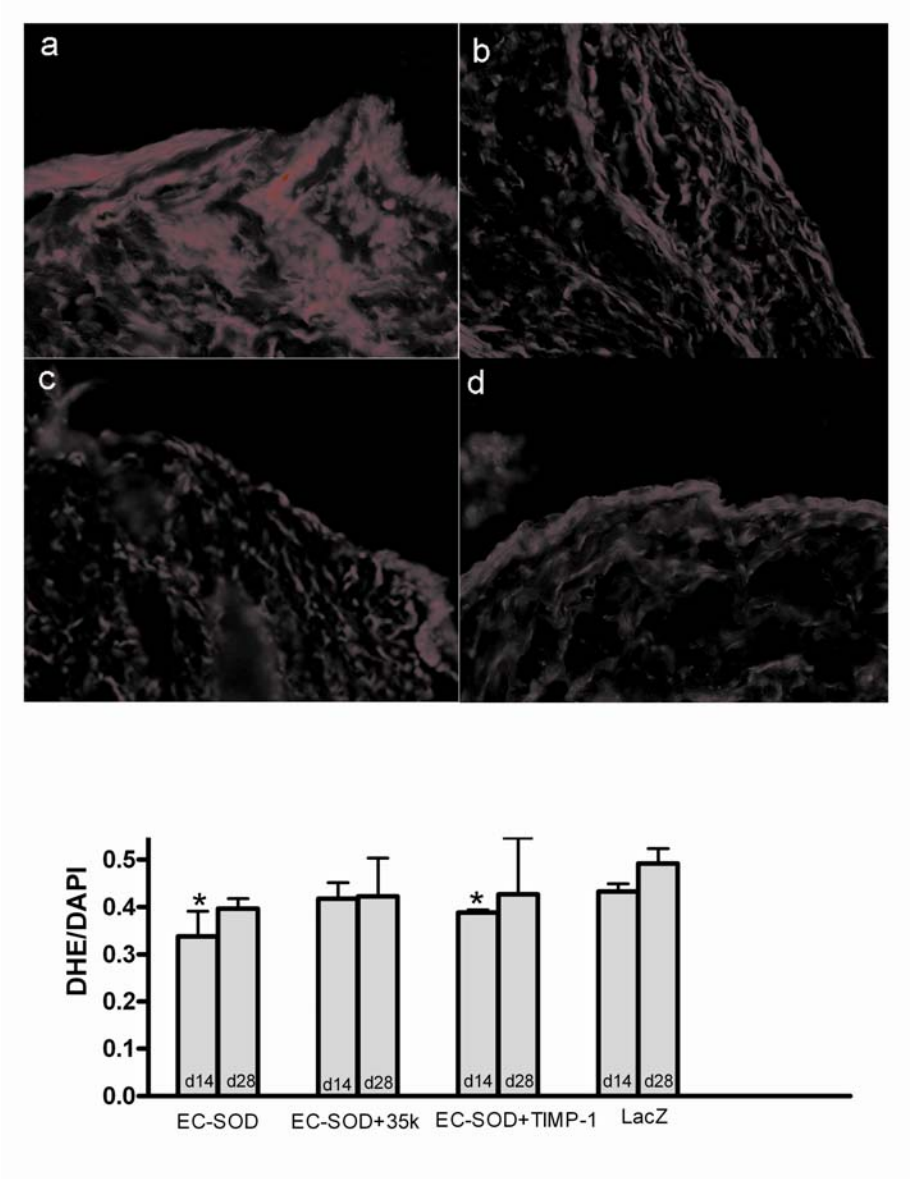

Figure 4. Representative examples of the production of superoxide anion in vein grafts as analyzed by DHE staining two weeks after the gene transfer. a) AdLacZ-treated rabbits show many scattered fluorescence-positive areas; b)Ad(EC-SOD+35k), c) AdEC-SOD d) Ad(EC-SOD+TIMP-1) groups showed decreased staining compared to AdLacZ control group suggesting the presence of lower oxidative stress in study groups. Magnification x200. Graph below: superoxide anion production in the vessel wall was determined by DHE assay two weeks and four weeks after the gene transfer. The number of total nuclei was counted after DAPI application and the values were calculated as a ratio of DHE/DAPI. *P<0.05.

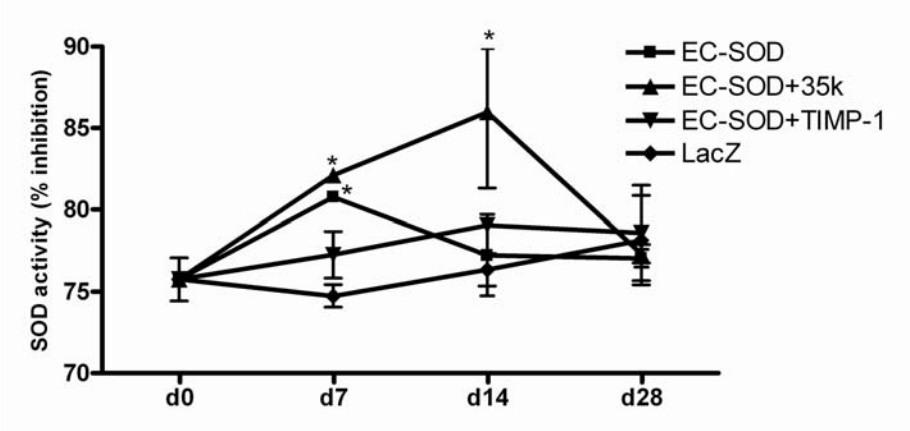

Figure 5. Total plasma SOD activity was increased significantly in AdEC-SOD and Ad(EC-SOD+35k) groups 7 and 14 days after the gene transfer. Values are mean±SEM, *P<0.05.

a) **2 wk** positive staining	EC-SOD	EC-SOD+35k	EC-SOD+TIMP-1	LacZ
1-2 (low)	9	10	7	9
3-4 (high)	1	1	3	10
chi-test p	0.0245*	0.0171*		
b) **4 wk** positive staining	EC-SOD	EC-SOD+35k	EC-SOD+TIMP-1	LacZ
1-2 (low)	6	10	7	1
3-4 (high)	1	1	2	7
chi-test p	0.005**	0.0006**	0.007**	

Table 1. Apoptosis as detected by positive immunohistochemical staining (low staining scores: (1-2), high staining scores (3-4)) Chi-square test. **P<0.005 in comparison to the AdlacZ group at the same time point.