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Gene Therapy and Atherosclerosis

Experimental Studies With
Secreted Macrophage Scavenger Receptor and
Platelet Activating Factor Acetylhydrolase

Doctoral dissertation

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Kuopio for public examination in Auditorium of Kuopio University Hospital,
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ABSTRACT

Atherosclerosis is initiated by an injury to the endothelium that results in low density lipoprotein (LDL) extravasation to the vessel wall where it is oxidatively modified. Oxidized LDL (OxLDL) attracts blood monocytes to enter subendothelial space where they become tissues macrophages, endocytose modified LDL, become lipid laden foam cells and cause fatty streak formation.

Human macrophage scavenger receptors type A I/II (hMSR) mediate modified lipoprotein uptake and foam cell formation. It has been shown that deletion of the MSR A I/II gene inhibits atherosclerosis in ApoE knock-out mice.

Oxidative stress leads to the generation of platelet activating factor (PAF) and oxidized phospholipids with PAF-like bioactivity in LDL. PAF acetylhydrolase (PAF-AH) catalyzes the hydrolysis of PAF and PAF-like oxidized lipids and therefore protects LDL from oxidative modification. However, a reaction product of the hydrolysis is lyso-PAF that is atherogenic.

In the present study we investigated the possibility to affect scavenger receptor mediated functions *in vitro* by using secreted macrophage scavenger receptor (sMSR). We also studied the possibility to inhibit atherosclerosis in mice by virus mediated gene transfer of sMSR. In addition, we studied the possibility to inhibit LDL oxidation and foam cell formation by PAF-AH gene transfer.

It was found that the soluble scavenger receptor inhibited the degradation of modified LDL in wild type and in MSR A I/II knock-out murine macrophages by 70%. Furthermore, sMSR inhibited foam cell formation in macrophages and monocyte/macrophage adhesion to endothelial cells *in vitro*. *In vivo* adenovirus mediated sMSR gene transfer inhibited about 40% of the MSR AI/II activity and inhibited atherosclerotic lesion formation by 14-19% in hypercholesterolemic LDL receptor (LDLR) knock-out mice. In a study where a long term effect of sMSR was studied, AAV mediated gene transfer of sMSR reduced aortic atherosclerosis by 21% in hypercholesterolemic LDLR knock-out mice without adverse effects. It was also found that adenovirus mediated gene transfer of PAF-AH into rabbit liver caused a production of functional PAF-AH that was associated to LDL and protected rabbit LDL from oxidation which lead to reduced foam cell formation.

It is concluded that sMSR is a useful tool in studying the macrophage scavenger receptor function *in vitro* and *in vivo*. In addition, affecting modified lipoprotein receptors and modified lipoprotein metabolism with sMSR is feasible and results in reduced atherosclerosis in a mouse model of atherosclerosis. It is also concluded that lipoprotein oxidation and atherogenic properties can be inhibited by using the enzyme PAF-AH.

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ABBREVIATIONS

AAV	Adeno-associated virus
AcLDL	Acetylated LDL
Ad	Adenovirus
Ag	Antigen
AGE	Advanced glycation endproducts
Apo	Apolipoprotein
AT	Angiotensin
bFGF	Basic fibroblast growth factor
cDNA	Complementary DNA
cfu	Colony forming unit
CHD	Coronary heart disease
CMV	Cytomegalovirus
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
FIV	Feline immunodeficiency virus
FH	Familial hypercholesterolemia
GM-CSF	Granulocyte-monocyte colony stimulating factor
HDL	High density lipoprotein
HERV	Human endogenous retrovirus
hMSR	Human macropahge scavenger receptor
HSA	Human serum albumin
ICAM-1,2	Intercellular adhesion molecule 1 and 2
IFN- γ	Interferon gamma
IGF-1	Insulin like growth factor 1
IL-1	Interleukin-1
ITR	Inverted terminal repeat
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LDLR ko	Low density lipoprotein receptor knock-out
Lp-PLA ₂	Lipoprotein associated phospholipase A ₂
LPS	Lipopolysaccharide
LTR	Long terminal repeat
LOX-1	Lectin like receptor for oxidized LDL
M-CSF	Monocyte colony stimulating factor
MDA	Malondialdehyde modified LDL
MHC-complex	Major histocompatibility complex
m/pAb	Monoclonal / polyclonal antibody
MM-LDL	Minimally modified LDL
MMP	Matrix metalloproteinase
MMuLV	Moloney murine leukemia virus
MOI	Multiplicity of infection
MSR	Macrophage scavenger receptor
NF- κ B	Nuclear factor κ B
NLS	Nuclear localization signal
NK	Natural killer cell

NRP-1	Neuropilin-1
OxLDL	Oxidized LDL
PAF-AH	Platelet-activating factor acetylhydrolase
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PFU	Plaque forming unit
PMN	Polymorphonuclear leukocytes
PTCA	Percutaneous transluminal coronary angioplasty
RAGE	Receptor for advanced glycation endproducts
RCR	Replication competent retrovirus
RGD	Arginine-Glycine-Aspartic acid
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-Page	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SMC	Smooth muscle cell
SCID	Severe combined immunodeficiency
SD	Standard deviation
SEM	Standard error of mean
SIV	Simian immunodeficiency virus
sMSR	Soluble macrophage scavenger receptor
TGF- β	Transforming growth factor beta
TIMP	Tissue inhibitor of matrix metalloproteinases
TNF- α	Tumor necrosis factor alpha
TLR	Toll-like receptors
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VSV-G	Vesicular stomatitis virus G protein

LIST OF ORIGINAL PUBLICATIONS

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

- I Laukkanen, J., Lehtolainen, P., Gough, P., Greaves, D.R., Gordon, S., Ylä-Herttuala, S. Adenovirus-mediated gene transfer of a secreted form of human macrophage scavenger receptor (SR-A1) prevents modified LDL degradation and foam cell formation in macrophages. *Circulation*, 2000; 101:1091-1096
- II Jalkanen J, Leppänen P, Närvänen O, Greaves DR, Ylä-Herttuala S. Adenovirus-mediated gene transfer of a secreted decoy human macrophage scavenger receptor (SR-AI) in LDL receptor knock-out mice. *Atherosclerosis* 2003; 169:95-103
- III Jalkanen J, Leppänen P, Pajusola K, Närvänen O, Mähönen A, Vähäkangas E, Greaves DR, Bueler H, Ylä-Herttuala S. Adeno-associated virus -mediated gene transfer of a secreted decoy human macrophage scavenger receptor (SR-AI) reduces atherosclerotic lesion formation in LDL receptor knock-out mice. *Molecular Therapy*, 2003;8(6):903-910
- IV Turunen P, Jalkanen J, Heikura T, Puhakka H, Ylä-Herttuala S. Effect of Adenovirus-mediated Gene Transfer of Platelet Activating Factor Acetylhydrolase on LDL Oxidation and Foam-cell Formation. *Submitted* 2004

In addition, some unpublished data are presented

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1 INTRODUCTION

Atherosclerosis is a progressive disease and the underlying cause of clinical conditions including coronary heart disease (CHD) and peripheral vascular disease. It is a common cause of morbidity and mortality in developed countries and therefore a subject of intensive research.

Over 25% of all deaths with both genders are caused by CHD in Finland (Salomaa et al., 1996). The three most important risk factors for CHD are high low density lipoprotein (LDL) concentration, hypertension and smoking (Anderson et al., 1991). They predict - not only the onset of CHD - but also new cardiovascular events with patients already suffering from CHD. In addition, male gender, age over 45 with men and over 55 with women, low high density lipoprotein (HDL) (or ratio of total cholesterol to HDL), diabetes and left ventricular hypertrophy are known risk factors for CHD. Genetic factors predispose patients to CHD and many patients have a long family history of cardiovascular disease.

The conventional treatment of CHD includes elimination of the known risk factors and treating the established disease with anti-ischemic and antithrombotic drugs. If ischemia persists despite of the medical treatment balloon angioplasty (PTCA), stenting or by-pass surgery can be performed. However, PTCA, stenting and by-pass surgery are palliative interventions that do not correct the underlying pathology.

The development of molecular techniques have taken the study of diseases and their treatments to the cellular level and made gene therapy an attractive candidate to treat CHD and peripheral vascular disease without affecting the healthy cells of the body. A number of different genes have been tested in animal models and an increasing number of genes have been taken to clinical trials. However, problems associated with vector development remain a limiting factor in gene therapy.

Soluble receptors are a newly discovered tool in gene therapy. Soluble receptors are secreted molecules that compete with the transmembrane receptor for ligand binding and therefore antagonize receptor mediated functions. Since receptors are secreted the therapeutic protein is spread beyond the transfected cells broadening its therapeutical potential.

An important receptor in the pathogenesis of atherosclerosis is the macrophage scavenger receptor AI (MSR). It is a lipoprotein receptor capable of mediating foam cell formation. It has been shown that deletion of MSR A I/II gene in mice decreases the rate of atherosclerosis. Therefore MSR is a potential target gene for gene therapy.

Platelet activating factor acetylhydrolase (PAF-AH) that is also called lipoprotein-associated phospholipase A₂ (Lp-PLA₂) is an enzyme capable of catalyzing the hydrolysis of platelet activating factor (PAF), bioactive phospholipids and PAF-like lipids and may therefore be capable of protecting LDL from oxidation. On the other hand, it shows a strong correlation to the risk of atherosclerosis.

The purpose of this study was to create a soluble macrophage scavenger receptor (sMSR) that would function as an antagonist to the transmembrane scavenger receptor. We aimed to evaluate whether sMSR could inhibit MSR mediated functions and if so, whether inhibition of MSR activity by systemic *in vivo* gene transfer could inhibit atherosclerosis in a mouse model of atherosclerosis. In addition, we wanted to explore the role of PAF-AH in LDL oxidation and foam cell formation.

2 REVIEW OF THE LITERATURE

2.1 Pathogenesis of atherosclerosis

Atherosclerosis is common cause of morbidity and mortality in developed countries. Types of atherosclerotic disease include CHD, peripheral vascular disease, abdominal aorta's aneurysm and carotid artery atherosclerosis. Atherosclerosis is a progressive disease that begins early in life – often already in childhood (Stary, 2000). In Finland 80% of the 15 year old children have fatty streaks in their coronary arteries and about 40% have fibrous plaques (Ylä-Herttuala et al., 1986). Clinical manifestations of atherosclerosis begin to appear in fifth and sixth decade of life and the frequency and severity of the complications correlate with the number of known risk factors, including hyperlipidemia, hypertension, diabetes, smoking and family history of atherosclerotic cardiovascular disease.

According to the current consensus on the origin of atherogenesis, injury to the endothelium appears to be the catalyst that begins the development of atherosclerotic lesions. Hypercholesterolemia is the main contributing factor to the development of atherosclerosis. It has been shown that for every 10% reduction in cholesterol level, coronary heart disease deaths are reduced by 15% (Steinberg, 2002). Hypertension, smoking etc. are additional risk factors that are unable to cause atherosclerosis alone but when combined to hypercholesterolemia can hasten the development of the disease.

Intact endothelium is of utmost importance to the normal function of an artery. It forms a non-thrombogenic and non-adherent surface for platelets and leukocytes, it actively transfers substances from the circulation to the vessel wall and vice versa, it maintains vascular tone, secretes growth factors and cytokines and it can modify lipoproteins. Injury to the endothelium impairs the function of the endothelium. Oxidized lipoproteins are thought to be essential in the initial endothelial insult that precedes lesion formation. The progression of atherosclerosis can be divided in three steps: *fatty streaks, intermediate lesions and advanced lesions* (Stary, 2000; Ross, 1999).

2.1.1 Fatty streaks

“Response to injury” -hypothesis first introduced by Ross and colleagues (Ross, 1993; Ross and Glomset, 1976; Ross, 1999) states that the atherosclerotic lesions are initiated in response to factors that cause injury or dysfunction to the arterial endothelium – such as sheer stress, inflammation, viruses and oxidized LDL (OxLDL) - particularly at the branch points of the arterial tree. Injury/dysfunction compromises endothelial cells' ability to act as a selective barrier and plasma constituents like lipids start to leak to the subendothelial space. In the arterial wall LDL binds to proteoglycans which leads to the locally increased LDL concentration. Proteoglycan bound LDL is more avidly modified by oxidation and glycation and these modified LDL particles are ligands for macrophage scavenger receptors. OxLDL further damages the endothelium and attracts monocytes and T-cell to the lesion area (Steinberg, 1997).

Formation of a chemotactic gradient that guides leukocytes to the site of inflammation is crucial to monocyte recruitment. Chemokines are potent activators and chemoattractants to leukocytes (Murdoch and Finn, 2000) and their expression is induced by a number of atherogenic stimuli including oxidized lipids, vascular injury, growth factors (such as platelet derived growth factor, PDGF) and cytokines (tumor necrosis factor alpha (TNF- α), interleukin-1 alpha (IL-1 α) and interferon gamma (IFN γ)) (Gerszten et al., 2000). Most

chemokines have a heparin binding site that allows them to attach to the proteoglycans. Therefore chemokines play an essential role in forming the chemotactic gradient.

Monocyte chemoattractant protein-1 (MCP-1) expression may be one of the initial steps of atherosclerotic lesion formation. MCP-1 is a chemokine that is expressed in the smooth muscle cells of balloon injured rabbit aortas only 2 hours after the injury, before macrophage accumulation can be seen. MCP-1 expression has been detected in the endothelial cells of early atherosclerotic lesions in humans. MCP-1 is expressed in macrophage-rich areas of human atherosclerotic lesions but also in smooth muscle cells and it is actively involved in the recruitment of new monocytes into the lesions (Nelken et al., 1991).

MCP-1 / LDLR double knock-out mice have about 80% smaller atherosclerotic lesions and MCP-1 deletion in the apolipoprotein B (ApoB) transgenic background about 70% smaller atherosclerotic lesions compared to controls. Lesions in these mice also contain fewer monocytes (Gosling et al., 1999; Gu et al., 1998). Similarly, crossing MCP-1 knock-out mice with ApoE knock-out mice results in 50% less lesions compared to ApoE single knock-out mice (Dawson et al., 1999). Overexpression of MCP-1 in ApoE knock-out mice leads to accelerated atherosclerosis due to the increased number of macrophages and increased lipid uptake in lesions. Furthermore, MCP-1 receptor is upregulated in hypercholesterolemic patients and LDL increases their receptor expression in monocytes (Han et al., 1998).

Injury to the endothelium increases adhesion molecule expression which permits blood monocytes, T-cells and platelets to adhere to the endothelium and enter the subendothelial space (Price and Loscalzo, 1999). Although there is some overlap in functions, the initial contact between leukocytes and endothelium is mainly mediated by the P- and E-selectins expressed by the endothelial cells and firm adhesion by vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). VCAM-1 binds to the $\alpha_4\beta_1$ integrin that is expressed in monocytes and lymphocytes. VCAM-1 expression is upregulated in endothelial cells in response to OxLDL, and VCAM-1 is expressed in lesion prone sites before the lesion development (Iiyama et al., 1999). VCAM-1 is considered important in early atherosclerosis (Cybulsky et al., 2001). Whereas VCAM-1 is quite specific to atherosclerotic lesions, ICAM-1 is more broadly expressed in the vasculature. It binds to the $\alpha_L\beta_2$ integrin expressed in leukocytes and may also be involved in lesion development although there are some contradicting results (Cybulsky et al., 2001). ICAM-1 may play a bigger role in the progression of the lesions (Collins et al., 2000; Bourdillon et al., 2000). Advanced glycation endproducts (AGE) found in diabetes increase the expression of both VCAM-1 and ICAM-1 via receptor for advanced glycation endproducts (RAGE) linking adhesion molecules also to diabetic atherosclerosis.

As monocytes enter the subendothelial space they differentiate into tissue macrophages and increase the expression of scavenger receptors. Macrophages accumulate modified lipids, become foam cells and along with T-cells form fatty streak lesions. Extracellular lipid may be present but the normal structure of the intima is not disorganized. Fatty streaks are normally not visible macroscopically (Stary, 2000).

2.1.2 Intermediate lesions and formation of fibroatheroma

Intermediate lesions and the role of smooth muscle cells, formation of fibroatheroma: As the lesion progresses the normal structure of the intima is replaced by a lipid core that contains extracellular lipids, cholesterol crystals and calcium. Macrophages, endothelial cells and smooth muscle cells (SMC) secrete growth factors and chemokines that attract more monocytes (MCP-1, macrophage-colony stimulating factor (M-CSF), and transforming

growth factor beta (TGF- β) from the circulation and SMC (PDGF, insulin like growth factor 1 (IGF-1)) from the media to the intima and they further modulate the lesion. SMC change from a contractile to a synthetic phenotype and contribute to the growing lesion by proliferating and secreting extracellular matrix. Peptide growth factors, excluding TGF- β , are positive regulators of the cell cycle enhancing migration and proliferation. They are regulators of differentiation and apoptosis (Heldin and Westermark, 1999; Waltenberger, 1997).

PDGF (especially PDGF-BB) is a mitogen for SMC and fibroblasts. PDGF protein and PDGF- β receptor expression can be detected in atherosclerotic plaques and PDGF- α receptors are expressed on injured endothelium (Lindner and Reidy, 1995; Ross, 1993). PDGF-BB injection causes neointima formation (Jawien et al., 1992) that can be inhibited by neutralizing antibodies against PDGF (Ferns et al., 1991). However, stimulation of migration rather than mitogenic activity may be the major role of PDGF in atherosclerosis.

Hypercholesterolemia increases PDGF A- and B- chain mRNA levels in mononuclear cells (Billett et al., 1996) an observation which links PDGF to cholesterol induced atherosclerosis. In addition PDGF-BB is able to enhance collateral artery formation and the formation of functional vascular anastomoses (Brown et al., 1995).

Similarly to PDGF, basic fibroblast growth factor (*bFGF*) injection causes neointimal thickening and inhibits apoptosis (Edelman et al., 1992) whereas inhibition of bFGF by antisense oligonucleotides inhibits early intimal thickening (Neschis et al., 1998) and induces apoptosis (Fox and Shanley, 1996).

TGF- β inhibits cell proliferation and migration and induces extracellular matrix production and apoptosis. Injection of TGF- β increases carotid artery neointimal thickness in balloon denuded rabbit arteries mainly by increasing extracellular matrix production (Kanzaki et al., 1995). Neutralizing antibodies to TGF- β given at the time of a balloon denudation inhibit intimal hyperplasia and fibrosis in rat aorta (Wolf et al., 1994). TGF- β isoforms are expressed in early human lesions and TGF- β receptors are upregulated in early fatty streaks (Bobik et al., 1999). However, in advanced lesions the expression of TGF- β and its receptors are located in isolated areas and the expression level of TGF- β receptor is reduced (Bobik et al., 1999; McCaffrey et al., 1999) indicating that cells are resistant to the antiproliferative effect of TGF- β .

2.1.3 Advanced lesion and plaque rupture

Eventually atherosclerotic lesions form a fibrous plaque that consists of a necrotic core filled with cholesterol esters and free cholesterol, covered by a cap of smooth muscle cells and extracellular matrix which occlude arterial lumen. It has been shown in restenosis that in the beginning of atherosclerosis the vessel wall can accommodate expansion of the neointima by dilating and therefore maintaining the lumen diameter. When the lesion mass reaches about 40% of the area encompassed by the internal elastic lamina, adaptive mechanisms are exceeded and further increase in the lesion mass results in the decreased lumen size and occlusion of the artery. Interestingly, lesions most prone to rupture are not lesions that occlude an artery. 68% of ruptured lesions causing a myocardial infarction are mildly stenotic (stenosis under 50%) (Falk et al., 1995). These lesions are also less likely to be visualized by angiography (Corti et al., 2002). Therefore, stable ischemia is due to the gradual occlusion of an artery whereas most coronary ischemic events are caused by rupture of an atherosclerotic lesion.

By morphology, atherosclerotic lesions can be classified as stable or vulnerable. Vulnerable plaques are prone to rupture and they cause the acute events in coronary heart disease. Vulnerable lesion contains a large lipid core – usually more than 40% of the whole lesion size - and a thin fibrous cap compared to stable lesions that have relatively small lipid core and thick fibrous cap. The shoulder regions that contain macrophages and T cells are especially prone to rupture which leads to hemorrhage and thrombosis and an acute coronary syndrome.

Macrophages secrete proteases and proteolytic enzymes like matrix metalloproteinases (MMPs) that degrade the extracellular matrix. T cells stimulate macrophages to produce metalloproteinases and may predispose plaques to rupture. T cells also inhibit SMC secretion of proteoglycans.

Angiogenesis in atherosclerotic plaque causes deleterious effects by facilitating plaque growth and causing intimal hemorrhages that may lead to arterial thrombosis. MMPs are required for degradation of basement membranes and extracellular matrix before migration and proliferation of endothelial cells and angiogenesis can occur. Endothelial cells secrete MMPs *in vivo* and anti MMP-2 and MMP-9 antibodies inhibit endothelial cell tube formation *in vitro*. In addition macrophages release cytokines that upregulate MMP expression in endothelial cells (George, 1998).

Atherosclerotic lesions develop in regions that are characterized by turbulent blood flow, and often localize to arterial bifurcations (Tricot et al., 2000). These sites are associated with increased endothelial cell turnover rates and apoptosis. Macrophages, T cells and SMC in atherosclerotic and restenotic lesions undergo apoptosis and the rate of apoptosis is more frequent in advanced lesions compared to fatty streaks. Myocardial ischemia and unstable angina pectoris have been associated with increased amounts of endothelial cell-derived membrane particles in plasma indicative of apoptosis (Mallat et al., 2000). In addition, SMC apoptosis is detected in primary atherosclerotic lesions as well as in restenosis. Apoptosis may be one factor influencing plaque rupture especially by weakening fibrous cap. Intimal accumulation of foam cells is associated with increased apoptosis and balloon denudation induces apoptosis. Therefore in a vulnerable plaque there is increased matrix breakdown and decreased synthesis of the fibrous cap.

Statins are effective lipid-lowering drugs but they can increase the diameter of a fixed stenosis only by tens of microns. However, they reduce the number of cardiovascular events by about 40% (1994) Thus they are far more effective drugs than could be interpreted from their lipid lowering effect alone. One proposed explanation could be an anti-inflammatory effect. In a rabbit atherosclerosis model a shift from high-cholesterol diet to low-cholesterol diet causes a disappearance of inflammatory cells in the lesions and the levels of collagenolytic enzyme (MMP-1) are reduced which can stabilize the plaque (Libby, 2000). In addition, improved endothelial function (O'Driscoll et al., 1997), reduced thrombogenic function (Bourcier and Libby, 2000) and an antioxidant effect (Rikitake et al., 2001) underlie the positive effect of statins (Liao, 2002).

2.1.4 Inflammation and atherosclerosis

Atherosclerosis is characterized by low-grade vascular inflammation. Thus, atherosclerosis can also be considered to be an inflammatory disease. Elevated levels of inflammatory mediators in healthy men and women have been shown to be associated with increased vascular risk. These inflammatory mediators include IL-6, TNF- α , cell adhesion molecules such as soluble ICAM-1, P selectin and E selectin, hsCRP, fibrinogen and serum amyloid A.

Lesions from fatty streaks to advanced plaques contain macrophages and T-cells and advanced human lesions contain antibodies to oxidized epitopes like OxLDL (Ross, 1999).

The immune system has two arms that collaborate with each other. Adaptive immunity depends on antigen-specific immunologic memory whereas innate immunity is characteristically antigen- and memory- independent. Cells and molecules that mediate the two immunological systems localize in atherosclerotic lesions and there is cross-talk between the two systems (Hansson et al., 2002; Libby et al., 2002).

Innate immunity is based on detection of pathogen-associated molecular patterns (PAMPs) by PAMP receptors, which evokes a toxic and inflammatory response. Innate immunity is fast, it can be activated in minutes to hours, but it is not adaptable and it is limited to germline encoded patterns. Innate immunity is characterized by cells belonging to the mononuclear phagocytic system especially macrophages, and natural killer cells (NK cells) and mast cells. Macrophages express receptors capable of recognizing molecular patterns foreign to mammals but common to many pathogens. Pattern recognition receptors including scavenger receptors (MSR) and Toll-like receptors (TLR) recognize PAMPs such as lipopolysaccharides (LPS), surface phosphatidylserine and aldehyde-derivatized proteins.

Ligation of PAMPs to macrophage scavenger receptors often leads to the endocytosis and lysosomal degradation of the particle whereas engagement of TLRs leads to activation of NF- κ B and mitogen activated protein kinase (MAPK) pathways leading to the activation of genes involved in leukocyte recruitment and, apoptosis, production of reactive oxygen species and production of cytokines that modify local inflammation. Atherosclerotic lesions express MSR A, CD36, CD68 and TLRs 1, 2 and 4 and to a lesser extent TLR 5 and 3. Expression of PAMP receptors colocalizes mainly to macrophages and endothelial cells (Edfeldt et al., 2002). TLRs are also a link between innate and adaptive immunity. Upon PAMP ligation on TLRs, dendritic cells digest proteins into small peptides and express them on their surface with MHC II complexes and present them to T cells leading to the T cell activation thus linking TLRs to both innate and acquired immunity.

Adaptive immunity recognizes unique molecular structures and depends on the generation of large numbers of T cell antigen receptors and immunoglobulins. Once T cells encounter an antigen presented in the context of an MHC molecule, an adaptive immune system is evoked. Cytotoxic T lymphocytes attack against the antigen bearing cells, B cells are stimulated to produce antibodies against the antigens and inflammation and innate immunity system is induced in the area where the antigen is present. The initial awakening of the adaptive immune system often requires help from the innate immune system. A dendritic cell, a mature form of Langerhans cell and the most potent antigen presenting cells, presents an antigen via MHC class II to T cells resulting in T cell activation. The adaptive immune system is slow, induction takes days. However, after the activation, the resulting memory T cells have a lower activation threshold. Macrophages and endothelial cells can present antigens and activate memory-effector T cells without dendritic cells.

The B cell's ability to produce antibodies against antigens results from somatic rearrangement of genes encoding the antibodies and hypermutations in the immunoglobulin genes. This affinity maturation creates antibodies that have increasing affinity to antigens. However, certain B cells do not undergo affinity maturation. They produce germline-encoded immunoglobulins that have a low affinity to antigens. Some of these natural antibodies recognize microbial components and some oxidized phospholipids of low-density lipoprotein (Shaw et al., 2000).

Immune deficient mice lacking T and B cells develop atherosclerotic lesions more slowly than immunocompetent mice (Emeson et al., 1996) and CD4⁺ T cell transfer into ApoE-SCID (severe combined immune deficiency) mice aggravates disease, implying that CD4⁺ T cells are a proatherogenic subset. On the other hand, elimination of T cells with monoclonal T cell antibody results in larger, proliferative lesions in balloon-denuded rat aortas and there is enhanced atherosclerosis in cyclosporine A-treated mice, in part due to the depletion of suppressor T cells (Emeson and Shen, 1993). Furthermore, class I MHC-deficient C57Bl/6 mice that lack cytolytic T cells and have impaired natural killer cell activity develop larger lesions in the aortic valve on high-fat diet.

B cells may protect against atherosclerosis by promoting Th1-inhibitory pathways. Hansson and co-workers (Caligiuri et al., 2002) have shown that transfer of either spleen cells or B cells to young splenectomized ApoE knock-out mice from atherosclerotic ApoE knock-out mice reduces atherosclerosis whereas splenectomy aggravates atherosclerosis. The disease aggravating splenectomy was associated with decrease in malondialdehyde modified LDL (MDA-LDL) antibody titer and the protective transfer of B cells was associated with an increase in the MDA-LDL antibody level.

OxLDL and inflammation: LDL is oxidized in the vessel wall where it serves as a substrate in foam cell formation. A small amount of OxLDL is also detected in the circulation.

OxLDL is chemotactic for monocytes and T cells (Quinn et al., 1987) and mitogenic for macrophages and SMC (Yui et al., 1993; Chatterjee and Ghosh, 1996). It is cytotoxic for endothelial cells (Hessler et al., 1983) and it stimulates the release of MCP-1 and of M-CSF from endothelial cells (Cushing et al., 1990; Rajavashisth et al., 1990). OxLDL induces the expression of VCAM-1, IL-1, IL-8 and MMP-1 and the activity of tissue factor-1. It mimics the effects of PAF (Marathe et al., 1999) and induces Fas mediated apoptosis (Sata and Walsh, 1998), it inhibits nitric oxide release or function (Murohara et al., 1994) and increases collagen synthesis in SMC (Jimi et al., 1995) and activates NF- κ B (Brand et al., 1997). Ligand uptake via MSR-A can lead to presentation of processed ligands to specific T cells linking the innate and adaptive immunity. T cell clones responsive to OxLDL have been isolated from human lesions (Steinberg, 1997; Steinberg, 2002).

Autoantibodies to OxLDL and MDA-LDL (an epitope of OxLDL) can be detected in the serum and they have been shown to be associated with atherosclerotic vascular disease. Autoantibody levels against MDA-LDL are greater in patients with rapid progression of carotid atherosclerosis and autoantibody levels are an independent predictor of the progression of carotid atherosclerosis in Finnish population (Salonen et al., 1992). In addition, increased autoantibody levels have been reported in patients with carotid atherosclerosis, coronary artery disease (Lehtimäki et al., 1999) and myocardial infarction (Vaarala et al., 1995), non-insulin dependent diabetes (Bellomo et al., 1995), peripheral vascular disease (Bergmark et al., 1995), hypertension and pre-eclampsia (Branch et al., 1994). In LDLR knock-out mice autoantibody titers correlate with the extent of aortic atherosclerosis. In another study, antibody titers to MDA-LDL correlate with lesion OxLDL content and progression of atherosclerosis in fat-cholesterol fed LDLR knock-out mice (Tsimikas et al., 2001). If normal chow is started after the high fat-cholesterol diet lesion OxLDL content is reduced. Also, IgG-OxLDL antibody titers positively correlate with intima/media thickness of the common carotid artery in clinically healthy, 58-year old men (Hulthe et al., 2001a) and serum OxLDL autoantibodies correlate with MMP-9 that may be involved in plaque rupture (Kalela et al., 2002). However, contradicting results have also been shown. When Uusitupa *et al* examined 91 NIDDM and 82 non-diabetic control patients in a

10-year prospective study they did not find and increase in the level of autoantibodies against OxLDL in NIDDM patients nor could autoantibodies predict atherosclerotic vascular disease in the common carotid artery of these patients.

However, oxidation specific antibodies and activation of cellular immune responses may have a protective role in atherosclerosis (Freigang et al., 1998). It has been shown that immunization with MDA-LDL and LDL reduces atherosclerosis in LDLR knock-out mice and WHHL rabbits and immunization with OxLDL and LDL reduces early atherosclerosis in NZW rabbits. In addition, immunization with OxLDL and LDL reduces neointimal formation after balloon injury in NZW rabbits. In man, hypercholesterolemic male patients had lower IgG titers compared with those in healthy controls. In the control group, there was an inverse correlation between intima-media thickness of the carotid artery bulb and IgM titers against OxLDL and MDA-LDL (Hulthe et al., 2001b).

It has been speculated that OxLDL antibodies and circulating OxLDL could form complexes that are rapidly cleared from the circulation. This would prevent OxLDL from penetrating arterial atherosclerotic lesions and exercising other atherogenic properties. In addition, antibodies penetrating lesions and complexing with OxLDL could be a target for uptake via macrophage Fc-receptor and again would prevent OxLDL mediated effects. The third mechanism could be activation of T cells via antigen presenting cells and MHC class II molecules leading modulation of the adaptive immune system.

2.2 Receptors for modified lipoproteins

LDL is an endogenous, liver-derived lipoprotein and it delivers cholesterol to the extrahepatic tissues. Of serum LDL 70% is cleared by the LDL receptor (LDLR) in the liver and extrahepatic tissues. The remaining 30% is cleared either unspecifically or by other lipoprotein receptors. Some of the LDL can be modified in the circulation or in the vessel wall and these modified LDL particles are ligands for the scavenger receptors. Members of the scavenger receptor family have been summarized in table 1. Discussed transgenic models are summarized in table 2.

2.2.1 Macrophage scavenger receptor class A

Macrophage scavenger receptors (MSR) class A type I, II and III are trimeric glycoproteins that are products of the same gene, generated by alternative splicing (Kodama et al., 1990; Rohrer et al., 1990; Goldstein et al., 1979). The predicted structures of the class A MSRs are depicted in figure 1. MSR A I contains a 110-amino acid C-terminal cysteine-rich sequence that is absent from MSR A II. MSR A I and II bind and internalize modified LDL (Kodama et al., 1990) including acetylated LDL (AcLDL), OxLDL and glycated LDL whereas MSR type A III is a dominant negative receptor that remains trapped within the endoplasmic reticulum (Gough et al., 1998). MSR A expression is upregulated by M-CSF and PMA that stimulate monocyte differentiation to macrophages whereas TNF- α , TGF- β , GM-CSF, IFN- γ and LPS decrease the expression in macrophages. MSR A I and II expression is upregulated in atherosclerosis (Ylä-Herttuala et al., 1991; Greaves et al., 1998a; Hiltunen et al., 1998; Naito et al., 1992; Suzuki et al., 1997). Because MSR A expression and activity are not down regulated by increasing cellular cholesterol content MSR A can mediate continuous accumulation of modified lipoproteins in macrophages and therefore cause foam cell formation (Kodama et al., 1990), one of the primary events in atherosclerosis. MSRs can also bind to matrix proteoglycans and thus anchor macrophages tightly to atherosclerotic lesions.

Long term exposure of proteins to glucose causes the formation of AGE. These molecules are increased in diabetes. AGE and AGE-modified LDL are ligands for MSR and incubation of AGE-LDL induces foam cell formation (Vlassara et al., 1985; Takata et al., 1988; Jinnouchi et al., 1998). In addition, MSR is able to bind to glucose modified collagen IV (el Khoury et al., 1994). This indicates a role of MSR in the accelerated diabetic atherosclerosis.

In addition to being a lipoprotein receptor MSR A has multiple other functions. It has been shown to cause cation independent adhesion of macrophages to cell culture plastic (Fraser et al., 1993) as well as to apoptotic thymocytes (Platt et al., 1999; Terpstra et al., 1997), B cells (Yokota et al., 1998), microglia and β -amyloid fibrils. MSR A can bind lipoteichoic acid from Gram positive bacteria (Dunne et al., 1994) and endotoxin from Gram negative bacteria (Hampton et al., 1991). Endotoxin up-regulates TNF α which causes vasodilatation followed by septic shock. MSR A knock-out mice are more susceptible to lethal endotoxic shock compared to wild type mice (Suzuki et al., 1997; Haworth et al., 1997).

Peritoneal macrophages from MSR A knock-out mice have only 20% of the ligand binding activity to AcLDL (Lougheed et al., 1997), 40-70% to OxLDL (Lougheed et al., 1997) and 30% to glycated LDL. Even though the binding and uptake of modified LDL is decreased in MSR A knock-out mice, turnover of OxLDL or AcLDL injected in these mice is comparable with the wild type mice indicating involvement of other modified LDL receptors in ligand uptake (Ling et al., 1997).

MSR A I and II knock-out mice have decreased atherosclerosis and MSR A I and II knock-out mice have been crossed to several different atherosclerotic mice models in order to further evaluate the significance of MSR A in lesion formation. In ApoE knock-out mouse background MSR A depletion causes about 60% fewer lesions compared to ApoE single knock-out mice (Suzuki et al., 1997). In LDLR knock-out mouse MSR A depletion causes a modest 20% decrease in atherosclerosis (Sakaguchi et al., 1998). Nevertheless, both double knock-out mice have foamy macrophages in atherosclerotic lesions indicating that A I and II are not the only receptors capable of mediating foam cell formation. In contrast to ApoE and LDLR knock-out mice, depletion of the MSR A activity in ApoE3Leiden transgenic mice results in the development of more complex lesions as judged by their cellular composition (de Winther et al., 1999), MSR A over-expression in bone marrow-derived cells does not increase atherosclerosis or foam cell formation in ApoE knock-out mice (van Eck et al., 2000) and MSR A transgene in LDLR knock-out mice reduces atherosclerosis (Wolle et al., 1995). The latter two observations can be explained by clearance of ApoB containing modified lipoproteins from the circulation by MSR A expressed in the liver. In addition, MSR A overexpression increases HDL cholesterol levels, decreases cholesterol esters in the liver and increases fecal bile acid flux.

MARCO

Macrophage receptor with a collagenous structure (MARCO) belongs to the class A scavenger receptor family. It is a coiled homotrimer that possesses scavenger receptor cysteine rich domain and an extended collagenous domain but lacks the α -helical coiled coil domain of MSR A. MARCO is expressed in subsets of macrophages in the peritoneum, marginal zone of the spleen and the medullar cord of lymph nodes. Inflammatory stimuli can increase the expression of MARCO in the liver. It is able to bind AcLDL and both Gram negative and Gram positive bacteria. It serves as a bacterial binding receptor and most likely plays a role in host defense. Most likely MARCO is not involved in atherosclerosis (Elomaa et al., 1995).

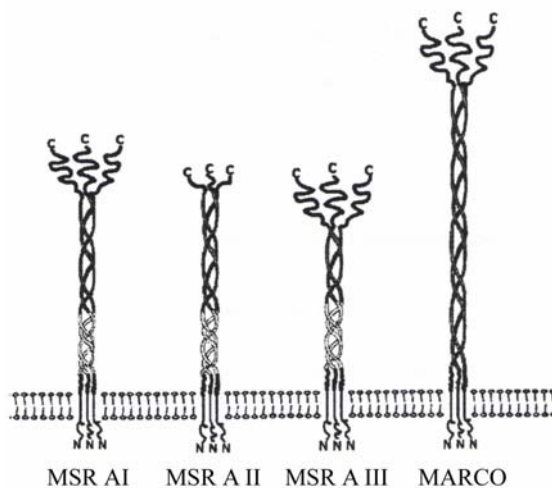


Figure 1. Predicted structures of the class A scavenger receptors. Picture modified from (Terpstra et al., 2000)

2.2.2 Scavenger receptor class B CD36

CD36 is a hydrophobic membrane glycoprotein found in platelets, erythrocytes, endothelial cells, B cells, macrophages and many tumor cells (Silverstein and Febbraio, 2000). It belongs to the scavenger receptor class B proteins and can bind long chain fatty acids, HDL, OxLDL, thrombospondin and apoptotic cells and mediate adhesion (Endemann et al., 1993). *CD36* expression increases as monocytes differentiate into macrophages and also in response to proatherogenic cytokines like IL-4 and M-CSF (Huh et al., 1995; Huh et al., 1996; Yesner et al., 1996). Some inflammatory mediators like LPS down-regulate *CD36* expression. Of particular interest is the observation that *CD36* expression is increased as cells accumulate OxLDL resulting in a potential to form foam cells (Huh et al., 1996). Another important feature is that, unlike MSR class A I and II, *CD36* can also bind and internalize minimally modified LDL (MM-LDL) which shows many of the atherogenic properties of OxLDL but probably has a longer half-life in the circulation. Furthermore, after MSR mediated binding to surface coated with OxLDL macrophages secrete H_2O_2 that can further oxidize LDL and is toxic to cells. This oxidative burst is at least partly mediated by *CD36* (Maxeiner et al., 1998).

In addition to being a proatherogenic scavenger receptor *CD36* has some antiatherogenic properties. *CD36* mediates the antiangiogenic properties of thrombospondin-1 (Dawson et al., 1997; Jimenez et al., 2000) and blocking angiogenesis in vessel wall inhibits plaque growth (Moulton et al., 1999). Clearance of apoptotic cells (Ren et al., 1995) limits the tissue damage by toxic intracellular agents and increases plaque stability.

The uptake of copper modified LDL and LDL modified by leukocyte reactive nitrogen species is reduced by 60% and 70%, respectively in ApoE and *CD36* double knock-out mouse macrophages. These double knock-out mice also have 80% less lesions in Western type diet compared to ApoE single knock-out mice (Febbraio et al., 2000). In addition *CD36* expression has been detected in foam cells of human atherosclerotic lesions (Nakata et al., 1999).

CD36 deficiency has been detected in humans (Yamamoto et al., 1990; Curtis and Aster, 1996). Macrophages isolated from individuals that are completely defective for *CD36*, bind

and internalize 40% less OxLDL compared to non-defective individuals (Podrez et al., 2000). CD36 deficiency has been associated with impaired cardiac function. In Japan 38% of the patients with hypertrophic cardiomyopathy were CD36 defective which may be due to the impaired fatty acid transport in the myocardium (Tanaka et al., 1997). This may limit the possible future therapies designed to block CD36 activity.

SR-BI and II

Another important class B scavenger receptor is the *scavenger receptor B I* (SR-BI) (Acton et al., 1996). It is a HDL receptor that can also bind apoB containing lipoproteins like LDL (Acton et al., 1996), anionic phospholipids (Rigotti et al., 1995) and senescent or apoptotic cells (Rigotti et al., 1995). SR-BII is an isoform of the same gene as SR-BI but SR-BII mRNA is less efficiently translated. Both SR-BI and SR-BII mediate selective uptake of HDL but SR-BII is 4 times less efficient (Acton et al., 1996).

SR-BI is highly expressed in the liver and steroidogenic tissues like the adrenal gland and ovary and in the mammary gland of a pregnant rat. Low levels of SR-BI expression can be detected in unstimulated testis and intestine. Hepatic lipase is found in the tissues that express high levels of SR-BI (Bensadoun and Berryman, 1996) and it can affect the metabolism of HDL and other lipoproteins by stimulating the delivery of cholesterol from HDL (Bamberger et al., 1983; Kadowaki et al., 1992). It has been noted that hepatic lipase deficient mice have altered SR-BI mRNA expression (Wang et al., 1996). Therefore it is possible that SR-BI and hepatic lipase act together in selective HDL cholesterol ester uptake.

Hepatic overexpression of SR-BI leads to the virtual disappearance of HDL from the circulation and subsequent increase in the biliary cholesterol after adenovirus mediated gene transfer of SR-BI gene (Kozarsky et al., 1997). A similar change in HDL concentration has been obtained from transgenic mice overexpressing SR-BI in the liver. In addition, transgenic mice have a decrease in non-HDL lipoproteins and also a decrease in plasma apoA-I levels, possibly because of the increased hepatic uptake of HDL lipids and subsequent clearance of HDL apoA-I by the kidney (Ueda et al., 1999). Furthermore, the SR-BI transgene decreases atherosclerosis (Arai et al., 1999). When mice expressing high or low levels of SR-BI transgene were crossbred to human apoB transgenic mice it was found that mice expressing low levels of SR-BI had decreased atherosclerosis whereas mice expressing high levels of SR-BI had similar level of atherosclerosis to apoB transgenics but increased atherosclerosis compared to low SR-BI/apoB mice (Ueda et al., 2000). Adenovirus mediated gene transfer of SR-BI to LDLR knock-out mice reduces atherosclerosis possibly due to the increase in the reverse cholesterol transport from the peripheral tissues to the liver and secretion of the cholesterol to the bile (Kozarsky et al., 2000).

SR-BI knock-out mice have also been generated (Rigotti et al., 1997). The total cholesterol level of the homozygous SR-BI knock-out mice show about 2.2 fold increase in the total cholesterol concentration and the HDL particles are larger, more heterogeneous in size and contain more ApoE. In the SR-BI and apoE double knock-out mice the concentration of very low density lipoprotein (VLDL) and intermediate density lipoprotein (IDL) are increased and atherosclerosis is detected in the coronary arteries 5 weeks after birth – a time point of practically no lesion development in either of the single knock-out mice. Lesions are more human like and exhibit evidence of cholesterol clefts and fibrin deposition. ApoE / SR-BI double knock-out mice also develop spontaneous myocardial infarctions and cardiac dysfunction which have not been seen with previous mouse models of atherosclerosis

(Krieger, 1999; Braun et al., 2002). Therefore, SR-BI may primarily be antiatherogenic scavenger receptor.

2.2.3 Scavenger receptor class C

dSR-CI

dSR-CI is a cell surface receptor that mediates AcLDL uptake during *Drosophila* embryogenesis. When expressed in CHO cells dSR-CI mediated saturable binding, uptake and degradation of AcLDL. dSR-CI expression is restricted to haematocyte / macrophage lineage. It was isolated by expression cloning and shown to be a type I membrane protein of 609 amino acids (Pearson et al., 1995).

2.2.4 Scavenger receptor class D

CD68

The human *CD68* receptor and its murine homologue macrophage mannose receptor belong to the scavenger receptor class D receptors (Ramprasad et al., 1996). They are closely related to the lysosomal-associated membrane proteins (LAMP) that may have functions relating to cell-cell interaction or cell-ligand interaction. Almost all tissue macrophages express CD68. It is a highly glycosylated protein – glycosylation accounts for two thirds of its molecular mass – but also unglycosylated CD68 is able to bind OxLDL. CD68 and macrophage mannose receptor are predominantly intracellular proteins located in the late endosomes but small amount of protein is however expressed on the cell membrane. After the thioglycollate elicitation up to 15% of macrophage mannose receptor is expressed on the cell membrane of mouse peritoneal macrophages. Similar results have been obtained with human THP-1 monocytic cells: after PMA treatment 20% of the CD68 receptors are located on the cell membrane. CD68 is an OxLDL receptor and OxLDL binding can be inhibited by 30-50% with monoclonal antibodies to CD68 (Ramprasad et al., 1996). CD68 may have a role in the initiation of atherosclerosis since incubation of PMA stimulated THP-1 cells with OxLDL leads to the enhanced expression of CD68, CLA-1 and CD36 whereas the expression of MSR-A was unchanged and the expression of LOX-1 was down-regulated (Tsukamoto et al., 2002).

2.2.5 Scavenger receptor class E

LOX-1

Lectin like receptor for oxidized LDL (LOX-1) (Sawamura et al., 1997) shares the conserved structure of C-type lectin family and is able to bind and internalize oxidatively modified LDL but not native or AcLDL (Kume and Kita, 2001). Monoclonal antibody against LOX-1 is able to inhibit OxLDL binding to endothelial cells by 50-70%. LOX-1 expression is upregulated by angiotensin II in human coronary artery endothelial cells and it causes a concentration-dependent increase in the uptake of ¹²⁵I-labeled OxLDL by endothelial cells (Li et al., 1999). This uptake can be inhibited by AT₁ blockers but not by AT₂ blockers. Other inducers of LOX-1 expression are pro-inflammatory stimuli including TNF α , TGF- β and bacterial endotoxin as well as phorbol esters (Kume et al., 1998), OxLDL (Li et al., 1999), fluid shear stress (Murase et al., 1998), lysophosphatidylcholine (lyso-PC) (Aoyama et al., 1999), hypercholesterolemia and AT II (Li et al., 1999). LOX-1 is expressed in human atherosclerotic lesions (Kataoka et al., 1999) as well as in endothelial cells (Sawamura et al., 1997; Li et al., 1999), macrophages (Moriwaki et al., 1998) and activated vascular SMCs

Class	Receptor	Expression	Ligands	Function
A	SR-AI SR-AII SR-AIII	Kupffer cells, sinusoidal EC. Tissue MQ, foam cells	AcLDL, OxLDL, AGE-proteins, M-BSA, LPS, LTA, whole bacteria, poly(I), poly (G), β -amyloid	SR-A I / II: Endocytosis, phagocytosis, adhesion, binding and uptake of Gram-/+ bacteria, Ag presentation, apoptosis of thymocytes. SR-AIII: soluble form in the plasma.
	MARCO	Kupffer cells. MQ in marginal zones of spleen and lymph nodes,	AcLDL, LPS, whole bacteria	Endocytosis, phagocytosis. Binding and uptake of Gram-/+ bacteria
B	SR-BI/CLA-1 SR-BII	Hepatocytes, Kupffer cells. Monocyte/MQ, aderenal glands, testes, ovaries	HDL, LDL, VLDL, OxLDL, anionic phospholipids, apoptotic cells	Endocytosis, phagocytosis. IC delivery of cholesterol. Fatty acid transport, reverse cholesterol transport, phagocytosis of apoptotic T cells and damaged RBC, adhesion of monocytes
	CD36	Kupffer cells, sinusoidal EC, hepatocytes, hepatoma cells (HepG2). Monocyte/MQ, platelets, adipocytes, mammary epithelial cells, microvascular endothelial cells	HDL, LDL, VLDL, modified lipoproteins, anionic phospholipids, fatty acids, thrombospondin, collagen, <i>P falciparum</i> -infected RBCs, apoptotic cells	Endocytosis, phagocytosis Fatty acid transport, phagocytosis of apoptotic T cells and damaged RBC
C	Drosophila SR-C	Drosophila hemocytes / MQ	AcLDL	Endocytosis
D	CD68/ macrosialin	Kupffer cells. Tissue MQ	OxLDL, phosphatidylserine liposomes, malondialdehyde, BSA	Endocytosis (expressed mainly intracellularly)
E	LOX-1	Kupffer cells? EC? Vascular EC, MQ, SMC	OxLDL, carrageenan, poly(I), apoptotic cells	Endocytosis, phagocytosis, adhesion, binding of Gram-/+ bacteria, apoptosis of aged RBC, soluble Lox-1
F	SREC-I	Vascular EC, heart, placenta, lung, kidney, spleen, small intestine, ovary	AcLDL, OxLDL	Endocytosis, cell-cell interaction
	SREC-II		(AcLDL, OxLDL)	
Others	SR-PSOX	EC? Vascular EC	Vascular MQ (THP-1)	Endocytosis

Table 1. Expression and function of the scavenger receptor family. Table modified from Terpstra *et al* (Terpstra *et al.*, 2000). Abbreviations: MQ: macrophages, EC: endothelial cells, SMC: smooth muscle cells, RBC: red blood cell, IC: intracellular

(Kume et al., 2000). In early atherosclerotic lesions LOX-1 is mainly expressed in the endothelial cells but in more advanced lesions it is expressed in macrophages and SMC suggesting a role in foam cell formation. In a rabbit hypercholesterolemia model LOX-1 is expressed in the endothelium before accumulation of foam cells indicating that it may contribute to the activation of endothelial cells during early atherosclerosis (Chen et al., 2000). In addition to foam cell formation, LOX-1 mediates many other cellular functions: OxLDL binding to LOX-1 induces cellular oxidative stress and NF- κ B activation (Cominacini et al., 2000) and OxLDL-mediated upregulation of MCP-1 is LOX-1 dependent (Li and Mehta, 2000). LOX-1 supports cell adhesion to fibronectin (Shimaoka et al., 2001a) and to both Gram positive and Gram negative bacteria (Shimaoka et al., 2001b). LOX-1 mediated uptake of OxLDL by cultured human coronary artery endothelial cells induces apoptosis (Li et al., 1999) and OxLDL uptake by LOX-1 downregulates Bcl-1 and upregulates Bax leading to induced apoptosis in SMC (Kataoka et al., 1999).

In addition to the membrane bound LOX-1, LOX-1 has also a soluble isoform. A 35kDa soluble LOX-1 is formed by proteolytic cleavage that may involve serine proteases. Several membrane proteins have soluble counterparts that are sometimes agonists and sometimes antagonists to the membrane proteins. Soluble LOX-1 is the first soluble protein identified among scavenger receptor family. Further studies will be needed to determine whether its presence in circulation correlates to the atherosclerotic disease state and whether soluble LOX-1 can antagonize membrane LOX-1 activity.

2.2.6 Scavenger receptor class F

SREC-I and SREC-II

Scavenger receptor expressed by endothelial cells I (SREC-I) is an 830 amino acid class F scavenger receptor able to mediate uptake of AcLDL and OxLDL. SREC-1 also binds Poly I, dextran sulfate, MDA-LDL and M-BSA. SREC-II is an 834 amino acid peptide that has overall 35% homology with SREC-I (50% homology in extracellular regions and 20% homology in intracellular regions). In contrast to SREC-I SREC-II binds and internalizes AcLDL very weakly and does not bind OxLDL at all. Both SREC-I and II are expressed in heart, placenta, lung, kidney, spleen, small intestine and ovary. SREC-I and also to some extent SREC-II mediate a cell-cell interaction by their extracellular domains (homophilic trans-interaction). SREC-I and II show very strong heterophilic trans-interaction. Interactions are increased after incubation with AcLDL. Co-incubation with OxLDL abolishes heterophilic trans-interaction which led the authors to speculate that OxLDL has the ability to disrupt intercellular receptor/receptor interactions (Ishii et al., 2002).

2.2.7 Other scavenger receptors

SR-PSOX

Scavenger receptor for phosphatidylserine and oxidized lipoprotein (SR-PSOX) is a type I membrane protein that is expressed on human and murine monocyte / macrophages. SR-PSOX can bind and internalize OxLDL with comparable levels to MSR A. OxLDL binding is blocked by polyinosinic acid and dextran sulfate but not by AcLDL (Shimaoka et al., 2000). After cDNA transfection of COS-7 cells SR-PSOX is produced as a 27kDa precursor protein and 30kDa glycosylated protein. SR-PSOX is expressed in lipid laden macrophages in human atherosclerotic plaques in carotid endarterectomy and directional coronary atherectomy

specimens but not in normal arterial wall and therefore it may be important in the development of atherosclerosis (Minami et al., 2001).

Knock-out and transgenic mouse models	Lesions	References
ApoE / MSRA double knock-out	↓↓	(Suzuki et al., 1997)7)
LDLR / MSRA double knock-out	↓	(Sakaguchi et al., 1998)8)
Apo E Leiden tg / SRA knock-out	↑ (more severe lesions)	(de Winther et al., 1999)9)
ApoE / CD36 double knock-out	↓↓	(Febbraio et al., 2000)0)
SR-BI tg	↓	(Ueda et al., 1999; Arai et al., 1999)9)
hApoB tg / SR-BI high expression tg	↓↑	(Ueda et al., 2000)0)
hApoB tg / SR-BI low expression tg	↓	(Ueda et al., 2000)0)
ApoE / SR-BI double knock-out	↑↑	(Braun et al., 2002)2)
LDLR / MCP-1 double knock-out	↓↓	(Gu et al., 1998)8)
ApoBtg / MCP-1 knock-out	↓↓	(Gosling et al., 1999)9)
ApoE / CCR2 double knock-out	↓↓	(Dawson et al., 1999)9)

Table 2: Effect of reviewed genes on atherosclerosis.

2.3 PAF and PAF-AH/ Lp-PLA₂

Platelet activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, PAF) is a phospholipid autacoid with a glycerol backbone, an ether linked fatty alcohol at the 1-position, phosphocholine headgroup at the 3-position and a very short fatty acid acetate at the 2-position. PAF is produced by stimulated monocyte-macrophages, neutrophils, platelets, mast cells and endothelial cells. PAF is produced *de novo* from PAF-precursor phospholipid or by remodeling pathway from ether-linked phospholipids found as a membrane constituent. PAF produced by monocytes and macrophages is secreted whereas PAF produced by vascular endothelial cells is retained on the cell surface where it is involved in the early steps of inflammation and serves as a component (with the adhesion molecule P-selectin) of the signal that triggers the neutrophil binding to endothelial cells and the rapid extravasation of leukocytes and monocytes. PAF also causes platelet aggregation, vasodilatation, anaphylactic shock and increased vascular permeability. PAF stimulates monocyte secretion of cytokines and SMC growth. PAF activates platelets, neutrophils, monocytes, macrophages and vascular SMC. Therefore PAF has diverse physiological actions, particularly as a mediator of inflammation (Imaizumi et al., 1995; Prescott et al., 2000).

Many of the actions of PAF can be mimicked by structurally related phospholipids that are derived from oxidation (Prescott et al., 2000). PAF-like lipids are oxidized phosphatidylcholine derivatives generated from LDL by oxidation. The synthesis of PAF is tightly controlled while the synthesis of PAF-like lipids is not. The precursors of the synthesis contain polyunsaturated fatty acids that are susceptible to oxidative attack and fragmentation at the 2-position. This shortens the sn-2 residue and a carboxyl, hydroxyl or aldehydic oxygen function to the fragmented fatty acid chain can be added forming PAF-like lipids (figure 2). Some of these products resemble PAF and can be recognized by the PAF receptor and cause

PAF like activity. In addition, PAF-like lipids can exert their biological action distinct from the PAF receptor.

PAF acetylhydrolases (1-alkyl-2-acetyl-glycerophosphocholine esterase, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine acetylhydrolase, PAF-AH) are structurally diverse isoenzymes that catalyze the hydrolysis of the acyl group at the sn-2 position of glycerol in bioactive phospholipids and short residues of the PAF-like lipids. They constitute the group VI of *phospholipases A₂* (*PLA₂*) and have a specificity to short acyl chains. In addition to the secreted PAF-AH three types of intracellular PAF-AHs exist: isoforms Ia, Ib and II (Prescott et al., 2000). This text focuses on the secreted PAF-AH that is also called lipoprotein-associated phospholipase A₂ (Lp-PLA₂).

Strict substrate specificity is required to protect cell membranes and lipoproteins from continuous hydrolysis of the phospholipids. PAF-AH converts PAF to lysoPAF in a reaction that does not require calcium. Secreted PAF-AH is synthesized by macrophages, mast-cells and also hepatocytes but the plasma pool of PAF-AH most likely originates from hematopoietic cells (Asano et al., 1999). In plasma PAF-AH is associated to lipoproteins. Two thirds is associated to LDL and one third to HDL but only less than one percent of LDL contains PAF-AH. PAF-AH association with lipoproteins is pH dependent so that with pH 6 PAF-AH is transferred to HDL and at pH 8.5 to LDL. It has been shown that there are dissociable and nondissociable forms of PAF-AH that differ in their avidity to LDL and that the efficiency of hydrolysis of PAF is faster in LDL than in HDL (McCall et al., 1999; Stafforini et al., 1989). It has been proposed that PAF-AH in HDL serves as a reservoir of enzyme that is moved between LDL particles when needed and also that HDL serves as a shuttle that mediates the change of (dissociable) PAF-AH between LDL particles (Prescott et al., 2000). PAF-AH can also be associated lipoprotein(a).

PAF-AH is thought to exert a protective role and prevent LDL oxidation (Stafforini et al., 1992). However during the oxidation the activity of PAF-AH is decreased and oxygen radicals inactivate PAF-AH rapidly. Estrogen inhibits PAF-AH secretion by hepatocytes whereas glucocorticoids enhance its activity indicating that part of the anti-inflammatory action of glucocorticoids may be mediated by PAF-AH. The activity of PAF-AH decreases during the course of pregnancy and is at lowest level at gestation allowing the PAF to enhance the contraction of the uterus.

Variations in the plasma PAF-AH activity have been detected in various clinical conditions. Patients suffering from lupus erythematosus, necrotizing enterocolitis, sepsis or septic shock or severe bronchial asthma have decreased PAF-AH activity whereas patients with diabetes mellitus, essential hypertension or arthritis have increased PAF-AH activity. Patients with chronic cholestasis caused by liver diseases have elevated PAF-AH but PAFah level normalizes after liver transplantation (Yamada and Yokota, 1998).

During thrombotic events PAF is synthesized locally by endothelial cells. Endothelial cells also produce PAF in response to oxidative stress or various physiological agonists including thrombin, bradykinin and histamine and can induce monocytes to secrete superoxide anions. These factors indicate that PAF may contribute to the thrombotic events and development of atherosclerosis. In fact, it has been shown that the plasma PAF-AH activity is lower in men after acute myocardial infarction. In addition, about 4% of the Japanese population lacks plasma PAF-AH activity due to the single point mutation (G⁹⁹⁴→T transversion) in exon 9. This causes a Val²⁷⁹ → Phe substitution in the catalytic site of the PAF-AH. The frequency of the mutated allele was significantly higher in subjects with myocardial infarction than in control for men (but not for women) and the association was even more obvious with the patient group that lack the conventional risk factors indicating that the lack of PAF-AH is an

independent risk factor for CHD (Yamada and Yokota, 1998). In ApoE knock-out mice adenovirus mediated gene transfer of human PAF-AH reduced oxidized lipoproteins by 80%, macrophages by 69% and smooth muscle cells by 84% in the arterial wall compared to the ApoE knock-out mice transduced with control viruses. The resulted a 77% reduction in the neointimal area after injury-induced neointima formation. In addition, PAF-AH gene transfer reduced spontaneous atherosclerosis by 42% in male mice and 14% in female mice compared to controls (Quarck et al., 2001).

However, contradicting results have been presented. After LDL has been oxidized, oxidized phosphatidylcholines are degraded generating lysophosphatidylcholine (lyso-PC) and free oxidized fatty acids. The enzyme responsible for the degradation is PAF-AH. Lyso-PC has been shown to be very pro-atherogenic. It induces cytokine synthesis and migration of lymphoid cells and monocytes, it is chemoattractive for SMC, and it stimulates endothelial cell growth factor and VCAM-1 expression and induces inflammation and leukocyte accumulation after intracutaneous injection (Marathe et al., 2001). Some of the functions of lyso-PC are mediated through the PAF receptor but there is also a family of lyso-PC receptors. Accordingly, it has been shown that the activity of PAF-AH in plasma is increased in patients with atherosclerotic diseases such as myocardial infarction, peripheral vascular disease and ischemic stroke (Satoh et al., 1992). In addition, in the West of Scotland Coronary Prevention Study (WOSCOPS) it was shown that plasma levels of PAF-AH are an independent predictor of CHD (Packard et al., 2000). Also, atherosclerotic disease progression can be retarded in WHHL rabbits by using a PAF-AH inhibitor (Leach et al., 2001).

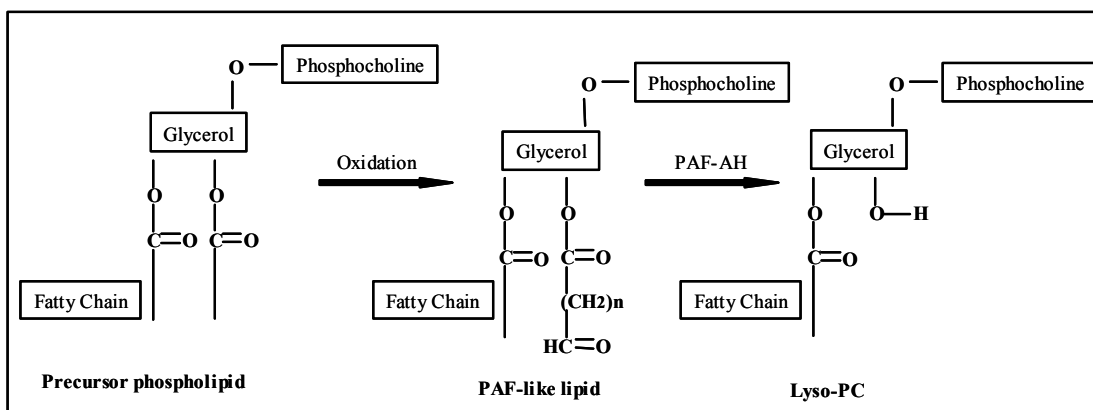


Figure 2. Formation of PAF-like lipid and lyso-PC from precursor phospholipid.

2.4 LDLR knock-out mouse

Mice are attractive candidates for research models because their genome has been sequenced and can be modified with relative ease, generation of mice is fast, they are easy to care for, small in size and cost-efficient. However, mouse lipoprotein metabolism differs significantly from human lipoprotein metabolism. A substantial amount of VLDL secreted by the liver contains ApoB-48 instead of ApoB-100 and remnants derived from ApoB-48 containing particles are cleared by the liver and not converted to IDL and LDL. Some of this clearance may be mediated by the chylomicron remnant receptors (ApoE receptor, LRP receptor). Also, most of the mouse plasma lipoprotein is HDL. Apart from C57BL/6 (B6)

mouse strain that develops fatty streaks on high fat diet, mice do not develop spontaneous atherosclerosis.

LDLR regulates the plasma cholesterol level by removing IDL and LDL that derive from VLDL synthesized in the liver. LDLR recognizes ApoE that is rich in IDL particles and apoB100 that is the main apolipoprotein in LDL. Humans deficient for LDLR suffer from familial hypercholesterolemia (FH) characterized by very high IDL and LDL concentrations, an increased conversion of IDL to LDL and an early onset of atherosclerosis (Goldstein and Brown, 1989)

Homozygous LDLR knock-out mice on chow diet have a two fold increase in the total cholesterol content compared to the wild-type litter mates without a significant change in the triglyceride levels. The increase in the serum cholesterol is mainly due to the increase in the IDL and LDL levels. IDL/LDL cholesterol is elevated by 7.4 fold and 9 fold in male and female mice, respectively. HDL-cholesterol content is elevated by 1.3 fold. In response to atherogenic diet the IDL/LDL fraction is elevated by three fold to 425mg/dl (11.05mmol/l). According to the changes in the lipoprotein profiles, the turnover of ¹²⁵I labeled lipoproteins is altered. The half life of VLDL is 10 min and 5 h and the half life of LDL is 2 h and 5 h in wild type mice and LDLR knock-out mice, respectively. The half life of HDL is unaltered. Adenovirus mediated gene transfer of a functional LDLR gene restores much of the wild type mouse lipoprotein profile and the turnover of VLDL is normalized (Ishibashi et al., 1993). Interestingly gene transfer of VLDL receptor (recognizes ApoE but not ApoB-100) results in an even more profound effect on lipoprotein profile possibly due to the fact that LDLR knock-out mice recognize LDLR as a neoantigen and this leads to the humoral and cellular immune response whereas ectopic expression of VLDL receptor does not induce an immune response (Oka et al., 2001). However, the elevation in the cholesterol levels of LDLR knock-out mice is moderate compared to the human FH patients. LDLR knock-out mice do not develop atherosclerotic lesions on chow diet but the lesion formation can be induced with atherogenic diets. After five to seven months on very high fat diet (1.25% cholesterol, 7.5% cocoa butter, 7.5% casein, 0.5% sodium cholate) LDLR knock-out mice develop conspicuous xantelasma, xanthomatous infiltration of the ears, ventral xanthomas and footpad thickening as well as massive atheromatous deposits within the proximal aorta. Atherosclerotic lesions appear early and are most profound in the root of the aorta and at the ostia of the coronary arteries with variable degree of xanthomatous change in the aortic valve leaflets. Lesions are also detected in the truncus pulmonalis but the distal coronary arteries remain intact.

In order to create a better mouse model for human hypercholesterolemia and atherosclerosis, LDLR knock-out mouse model has been modified in a number of ways. Some of the mouse models are summarized in the table 3.

2.5 Soluble receptors

Cytokines, growth factors and peptide hormones influence a great number of biological processes throughout the body and exert their function via membrane bound receptors. Some of these transmembrane receptors have a soluble counterpart. Soluble receptors can be formed by alternative splicing of the receptor mRNA or they can be generated by limited proteolysis (shedding) which cleaves the extracellular part of the transmembrane receptor off the cell membrane (Rose-John and Heinrich, 1994). There is no obvious similarity in the cleavage sites of different receptors, nor can any one particular protease be associated to shedding. Instead, PMA is a potent stimulator of many shedding processes indicating that activated protein kinase C is involved in shedding. Soluble proteins often differ from the

Mouse	Lipoprotein profile					Lesions	Ref
	Chol mg/dl (mmol/l)	VLDL	LDL	HDL	TG mg/dl (mmol/l)		
Wild type Chow (female - male)	100-119 (2.6-3.1)	-	-	-	119-133 (1.3-1.5)	Do not develop lesions	(Ishibashi et al., 1993)3)
ApoE ^{-/-} Chow	494 (12.8)	↑↑↑	↑	(↓)		On chow diet	(Palinski et al., 1994)4)
LDLR ^{-/-} Chow (female - male)	228-239 (5.9-6.2)	↑	↑↑		119-133 (1.3-1.5)	On high-fat or Western type diet	(Ishibashi et al., 1993; Sakaguchi et al., 1998)8)
LDLR ^{-/-} /ApoB-100Tg Chow (male – female)	745-853 (19.4-22.2)	↑	↑↑↑	↓	732-547 (8.3-6.2)	On chow diet	(Sanan et al., 1998)8)
LDLR ^{-/-} /ApoBec1 ^{-/-} Chow (male-female)	553-441 (14.4-11.5)		↑↑↑		208-125 (2.4-1.4)	On chow diet	(Powell-Braxton et al., 1998)8)

Table 3: Lipoprotein profiles in different atherosclerotic mouse models. **Western type diet** (21% fat (wt/vol), 0.15% cholesterol (wt/wt), 19.5% casein (wt/wt)), **High fat diet** (1.25% cholesterol, 20% casein, 1% corn oil, 0.5% cholic acid, 15% cocoa butter).

transmembrane proteins by missing some extracellular amino acids or having new amino acids in the C-terminus of the protein. New amino acids are due to the small deletions or insertions involving a reading frame shifts. The release of soluble proteins is stimulated by various physiological and pathophysiological conditions that change the homeostasis of the cell such as infections or inflammatory/autoimmune processes, some disease stages (Soluble LDL receptor-related protein, sLRP) and pregnancy (soluble vascular endothelial growth factor receptor FLT-1). In addition to cytokine and growth factor receptors, also other transmembrane proteins such as adhesion molecules or lipoprotein receptors have soluble counterparts.

Soluble receptors usually bind their ligand with similar affinity to that of the transmembrane receptor. Soluble receptors can function as carrier proteins for their ligands and protect the ligand against proteolytic degradation (Fernandez-Botran and Vitetta, 1991). They can also act as suppliers of cytokines/growth factors, they can desensitize cells and antagonize the function of the transmembrane receptor/growth factor-complex (IL-4 and TNF) (Maliszewski et al., 1990) or they can be agonists to the membrane bound receptors (Ozmen et al., 1993).

In gene therapy obtaining *in vivo* transfection efficiency sufficient for therapeutic effect is difficult with current vectors (see below). In this regard soluble/secreted molecules are advantageous because the therapeutic effect is not limited to the transfected cells. Therefore it is not surprising that both natural and synthetic soluble receptors have been used in different experimental settings.

RAGE is a receptor that specifically binds glycated proteins. Glycated proteins are formed in diabetes and also aging increases glycation. Uptake of glycated LDL by RAGE leads to foam cell formation. A truncated version for RAGE has been formed and administration of soluble RAGE protein (sRAGE) inhibits accelerated diabetic atherosclerosis (Park et al., 1998). In addition, RAGE is also a receptor for amphoterin, a polypeptide that has been linked to outgrowth of cultured cortical neurons. Transfection of sRAGE suppresses tumor growth and metastases in mice (Taguchi et al., 2000).

Levels of vascular endothelial growth factor (VEGF) are increased in pregnant women with pre-eclampsia. Recently it was shown that the placenta secretes soluble FLT-1 (sFLT-1), a soluble form of the VEGF-receptor FLT-1. It appears that sFLT-1 is needed to regulate VEGF levels and lack of regulation results in pre-eclampsia. Also, at least some malignant hematopoietic cells express sFLT-1 indicating that regulation of VEGF activity in normal and malignant hematopoietic cells may have an impact in the cell proliferation and tumorigenesis. Several solid tumors also secrete VEGF. Transfection of sFLT-1 to tumor cells before implantation inhibits tumor growth, metastasis and mortality rates in mice. Similar results were obtained with Neuropilin-1 (NRP-1), a receptor for VEGF₁₆₅. Interestingly, it was shown that NRP-1 and soluble NRP-1 were expressed in different tissues and that prostate carcinomas expressing recombinant sNRP-1 suffered from extensive hemorrhage, damaged vessels and apoptotic tumor cells. The results from pre-eclampsia and tumor studies show that soluble receptor for VEGF have an important role in regulating the effect of VEGF and that lack of regulation can lead to pathological conditions.

The IL-6 α receptor (IL-6R) (Modur et al., 1997; Galun et al., 2000) consists of two different proteins: IL-6 α and the homodimeric gp130. Gp130 is the transmembrane signaling protein and IL-6 α is the cytokine-binding protein. IL-6 α has a soluble counterpart that is formed both by shedding and by alternative splicing. Soluble IL-6 α is an agonistic receptor capable of mediating the effect of IL-6 to the cells that express only the gp130. The process is termed transsignaling. In mice a chimeric IL-6/IL-6R complex induced liver regeneration after severe hepatocellular injury when neither IL-6 nor IL-6R has been able to do so alone (Galun et al., 2000). In addition to IL-6 α receptor, gp130 also has a soluble counterpart that can antagonize IL-6 α activity by competing with the transmembrane gp130.

2.6 Gene therapy

The early idea of gene therapy focused on monogenic, inherited diseases by correcting or replacing defective genes. It was soon realized that correction of a mutation would be difficult and the concept of adding a functional gene in addition to the non-functional gene was created. From there on gene therapy has further broadened to cover acquired diseases such as cancer, atherosclerosis and neurological diseases. In fact, acquired diseases have become main targets of gene therapy development, because of the greater number of people they affect. Gene therapy can therefore be defined as a delivery of genetic material to tissues in order to treat diseases.

After initial enthusiasm it was realized that there is a number of obstacles before gene therapy can be a therapy of choice. 1) Our knowledge about the pathology of diseases is far from complete. Apart from monogenic diseases it is not always clear what genes would be most beneficial to be transferred and at which stage of the disease. 2) Efficiency problems have to be addressed. At the moment many of the conventional drugs are more efficient than gene therapy treatment. 3) Safety problems have to be solved. 4) Genetic models to test therapy are required before taking treatments to clinics and since “man is not a mouse” better genetic models are required.

2.6.1 Gene therapy of atherosclerosis

Familial hypercholesterolemia (FH) is an inherited disease caused by defects in the gene encoding LDL receptor. Homozygous, receptor negative patients suffer from severe hypercholesterolemia and an early onset of CHD. Receptor defective patients who have a

partially inactive LDLR have less severe hypercholesterolemia. Receptor defective patients may benefit from the treatment with statins but receptor negative patients are largely unresponsive to the statin treatment. Surgical interventions such as portacaval shunt and ileal bypass have resulted in transient lipid lowering effect. Plasmapheresis is effective but the patient is tight up to the treatment giving hospital and orthotopic liver transplantation requires life-long immunosuppressive medication. This is why gene therapy has been developed as a novel treatment strategy for FH.

First human gene therapy trial for FH has been performed in which five patients suffering from different mutations of the LDLR gene were treated with *ex-vivo* gene transfer of human LDLR. Although the patients endured the protocol well, the results were somewhat disappointing. Three out of five patients showed a prolonged effect in lipoprotein levels with up to 20% reduction in total cholesterol and 23% reduction in plasma LDL concentration. One patient had a small, insignificant reduction and one patient had no reduction in total cholesterol or LDL concentration. Interestingly, patients responded to the lovastatin treatment better than before the LDLR gene transfer and the effect was seen also with the patient that showed no response to gene transfer alone (Grossman et al., 1995).

VEGF₁₆₅ is an angiogenic factor that stimulates endothelial cell migration and proliferation, angiogenesis and accelerated endothelial repair. VEGF₁₆₅ may also be vasoprotective by causing endothelial cell production of NO and prostacyclin. VEGF₁₆₅ gene therapy has been tested for the treatment of CHD and atherosclerotic, peripheral vascular disease.

VEGF₁₆₅ gene transfer either with recombinant adenoviruses or plasmid/liposome complexes to human lower limb arteries led to increased vascularity in the treated limbs. However, gene transfer did not inhibit restenosis after percutaneous transluminal angioplasty (PTA) and there was no statistically significant effect on the outcome of hemodynamic studies among the groups (Makinen et al., 2002). Similar results were obtained in a study where a single, intramuscular dose of VEGF₁₂₁ expressing recombinant adenoviruses were used to treat patients suffering from peripheral artery disease. Gene transfer had no effect on exercise performance or quality of life (Rajagopalan et al., 2003).

In another randomized, placebo-controlled, double-blind phase II trial VEGF₁₆₅ adenoviruses, plasmid/liposome complexes and Ringer's lactate were compared in the prevention of postangioplasty and in-stent restenosis and in the treatment of myocardial ischemia. Gene transfer of VEGF₁₆₅ recombinant adenoviruses resulted in a significant improvement in regional myocardial perfusion. Gene transfer of VEGF₁₆₅ did not have an affect on the percentage of luminal stenosis or minimal lumen diameter as measured by quantitative coronary angiography. However, adenovirus mediated gene transfer of VEGF₁₆₅ was well tolerated. Gene transfer resulted in transient fever and a transient increase in CRP, LDH and adenovirus antibodies but no major adverse effects were detected (Hedman et al., 2003).

2.6.2 Viral gene transfer vectors

Viruses have evolved during millions of years and developed efficient methods to deliver their nucleic acids, escape the immunological surveillance of the host cell, propagate and release new virions. Therefore they are natural candidates for gene transfer vectors (Somia and Verma, 2000; Romano et al., 2000).

Viruses vary greatly in their abilities: retroviruses, adeno-associated viruses and lentiviruses integrate into the host cell genome and produce more or less stable expression while adenoviruses, baculoviruses and herpes simplex viruses mediate a transient gene expression.

Viral vectors also differ in immunogenicity. Some are almost immunologically inert while others raise a strong host immunological response. The size of genetic material that viral vectors can carry varies. It is also important that viruses can be produced with relative ease in large quantities and that the titers are sufficient for *in vivo* experiments. Since it is obvious that no single viral vector is efficient to solve all these problems, hybrid viruses containing elements from many different viruses have been generated.

Vectors that remain episomal are sufficient for diseases where only transient expression is needed to obtain therapeutic effect. When attempting to treat inborn genetic defects like FH, permanent expression is needed and integrating vectors have to be used. While permanent expression offers great possibilities, it also creates safety issues that have to be taken into consideration, and more advanced vectors are needed. It may be important for some diseases that the transgene is targeted and expressed only in the affected tissues. This can be achieved with tissue or cell type specific promoters although they have not turned out as good as originally was hoped for. Tissue specific promoters tend to “leak” and the expression that they drive is often weaker than with more general types of promoters. Tissue specific promoters also do not solve the problem of new genetic material spreading in the body; only the expression is limited to certain areas. Another approach is to add molecules such as peptide sequences (e.g. RGD) on the virus surface to target viruses to tissues. Physiological barriers such as internal elastic lamina, blood brain barrier or blood itself can limit the virus spread or inactivate the virus before it reaches its target. Therefore not only vectors but also methods to deliver the genetic material need to be improved. It is also necessary to have means to regulate the level of expression. This can be achieved for example with tetracycline or hypoxia inducible promoters that have already been tested *in vitro* and *in vivo* but it is also necessary to have means to shut down or delete the transgene if needed. This could be achieved by introducing a suicide gene or a cre-lox system in the vector construct. The problem is that often the size of the inserted cassette becomes too great and the stability and infectivity of the viruses decline. Currently used vectors are summarized in table 4.

2.6.2.1 Adenoviruses

Adenoviruses are oncogenic viruses that are also known to cause mild respiratory infections in humans. There are several different adenovirus serotypes, but Ad5 is most widely used for gene transfer purposes since it is not associated with malignancies. Adenoviruses are double-stranded, linear DNA viruses that have the ability to efficiently transfect both dividing and non-dividing cells. Adenoviruses mediate a strong but transient gene expression of about two to three weeks.

Adenovirus genome is 30-35 kb in size and consists of short inverted terminal repeats (ITR) that are the sites of onset of viral replication, four early genes (E1-E4), two delayed early units (IX, IVa2) and one late unit (major late) which is processed to generate five families of late mRNAs (L1-L5). Expression of adenoviral genes occurs in two phases relative to the onset of the adenoviral genome replication: early genes are activated before and late genes after genome replication. Early genes are needed for viral genome replication, interaction with the cell cycle and cell metabolism. E1 is the first transcriptional unit expressed and it is an absolute requirement for adenovirus replication. E1a is a transcriptional regulator responsible for transactivation of viral and cellular genes and transcriptional repression of other sequences leading to onset of genome replication. It also influences host cell cycle. E1b regulates the late mRNAs and together with E4 causes a blockage of the host protein synthesis. E2 encodes proteins necessary for virus replication, including DNA polymerase, DNA-binding protein

and precursor terminal protein and E3 region is involved in the cellular immunity. It is dispensable in terms of replication. E4 is involved in DNA replication, late mRNA synthesis, virion assembly and shutting off host protein synthesis. IX is required for packaging full-length genomic recombinants and late transcripts encode structural genes required for virus assembly (Berkner, 1988).

The E1 region has been removed from recombinant adenoviruses making them unable to replicate outside packaging cell line. Packaging cell lines such as 293, the human embryonic kidney cell line, complement E1 (E1a and E1b) in *trans*. A major disadvantage of these first generation adenoviruses is their immunogenicity due to the low amount of viral proteins expressed from recombinant vectors. Adenovirus transduction leads to the activation of CD4 (T-helper lymphocytes) and CD8 (cytotoxic T-lymphocytes) cells that destroy transduced cells as well as to the humoral response and production of antibodies making repeated administrations of adenoviruses inefficient.

An improvement to the vector system was the introduction of the temperature sensitive adenovirus. A mutation in the E2A-gene encoding the DNA binding protein makes viruses able to replicate at 32°C but not at physiological temperature 37°C therefore limiting viral protein expression. Indeed it has been shown that deletion of E2A decreases inflammatory response (Engelhardt et al., 1994) and prolongs expression of the transgene (Ye et al., 1996). However, factors that control the loss of transgene expression in the arterial wall appear to be different than in other organs, because the use of second generation adenoviruses does not result in improved transgene persistence in rabbit or mouse arteries (Wen et al., 2000).

The results from E1/E4 deleted adenoviruses have been even less clear. The rationale for the E4 deletion was that E4 region regulates viral gene expression at both transcriptional and posttranslational level, but while others reported longer transgene expression compared to E1 deleted viruses (Wang et al., 1997), others have not been able to show benefit from second generation viruses (Dedieu et al., 1997) or have reported detrimental effects. Reasons for these results could be the different mouse strains used and the differential route of administration of adenoviruses leading to differential expression.

An alternative approach to the design of adenoviral vectors is the use of E1 deleted helper viruses *trans*-complementing adenoviral sequences enabling deletion of adenoviral genes from the vector itself. This has been done with “guttled viruses”(Parks et al., 1996). The only *cis*-acting elements in the guttled viruses are the ITRs and packaging sequence. Viruses are propagated in 293 Cre cell line and helper viruses are rendered unpackageable by deleting packaging signal with cre-lox system. The use of these viruses has been reported to result in transgene expression for more than ten months with reduced acute and chronic toxicity. While one of the main aims with first and second generation adenoviruses was to produce non-replicating viruses and to avoid replication competent adenoviruses recently the idea of conditionally replicating adenoviruses (CRAd) has been introduced to tumor gene therapy (Alemany et al., 2000). The rationale behind this is that adenoviruses are naturally lytic viruses leading to the destruction of the infected cell and arousal of immune reactions that reduce the tumor size. Also the ability to replicate improves gene transfer efficiency. Currently two ways have been introduced to limit the infection. First, transcription of viral genes is controlled by replacing viral promoters with tumor-specific promoters and second, genes that have become dispensable in *tumor cells* have been deleted. An example of this is the tumor suppressor gene *p53*. *p53* shuts off the protein synthesis in the host cell after adenoviral infection but the viral E1b 55K gene product is able to bind and inactivate *p53* therefore enabling viral propagation. If 55K is deleted, adenoviruses replicate selectively in *p53* negative tumor cells. There are phase I and II clinical trials with 55K mutants but no effect have been seen in tumor growth.

	Baculo virus	Adenovirus	Retrovirus	Lentivirus (HIV)	AAV	HSV-1	Liposome
Cloning capacity	> 50kb	7-8 kb	8 kb	7 kb	4.5 kb	30 kb	> 20 kb (No theoretical limit)
Titer	10 ¹⁰ pfu/ml	10 ¹¹ pfu/ml	10 ⁵⁻⁸⁽⁹⁾ cfu/ml	10 ⁶⁻⁷ cfu/ml	10 ¹⁰ TU/ml	10 ⁴⁻⁸ cfu/ml	-
Preparation	Easy	Easy	Easy (Packaging cell line)	Four plasmid co-transfection	Easy	Relatively easy	Easy
Stability	Stable virus		Unstable, complement/ heat etc. inactivation (pseudotyped more stable)		Stable virus		Stable
Tropism	Broad					Neurotropic infects CNS cells, Broad with Pseudotyped	Broad
Efficiency	Very efficient		Low efficiency		Relatively efficient	Low efficiency	
Transduction ability	Non-dividing and dividing cells		Only dividing cells	Non-dividing and dividing cells		Non-dividing and dividing cells	Non-dividing and dividing cells
Expression	Transient	Episomal, transient (about 2-3 weeks). Can integrate with high titers.	Integration → stable expression		Integration (episomal) → stable expression	Does not integrate. Long-lasting/latent infection in neuronal cells.	Episomal transient
Immunologic reaction	Immuno-genic	Very immuno-genic (CTL, humoral). Wild type involved	Does not cause immunologic or toxic reactions	Non-immunogenic. Toxic at high titers	Does not cause immunologic or toxic reactions	Immunologic, inflammatory, cytopathic and toxic reactions.	Reactions to transgene / sequences of bacterial origin. Non-toxic.
Pathogenic	Non-pathogenic to humans	Mild upper respiratory tract infections	T-cell leukemia	AIDS	Non-pathogenic to humans	Stomatitis, (genital herpes)	Non-pathogenic
Adverse effects			Insertional mutagenesis. Possibility of recombination with human endogenous retroviruses. Homological recombination with the packaging cell line	Insertional mutagenesis. Possibility of recombination because of HIV-infection / serum conversion into HIV-1. Homological recombination because of overlapping genes.	Insertional mutagenesis		

Table 4: Properties of different gene transfer vectors.

Incorporation of HSV thymidine kinase suicide gene has been shown to improve therapeutic effect and safety of the CRAd viruses.

Adenoviruses mediated gene transfer is usually well tolerated in humans. However, one systemic inflammatory response leading to death of an 18-year old man suffering from partial ornithine transcarbamylase (OTC) deficiency has been encountered (Raper et al., 2003). Recombinant adenoviruses with E1/E4 deletion at a relatively high concentration of 6×10^{11} particles / kg were used. The patient suffered from systemic inflammatory response with disseminated intravascular coagulation and multiple organ system failure. This could not be anticipated from the earlier animal experiments. Therefore, the authors concluded that the safety data from different animal models may not be directly adaptable to men. Furthermore, similar dose of adenoviruses had been used with patients suffering from OTC deficiency. These patients experienced only flulike symptoms, transient rise in hepatic transaminases, thrombocytopenia and hypophosphatemia indicating that there may be substantial subject-to-subject variation in the response to adenoviruses. Lastly, recombinant adenoviruses have steep toxicity curve limiting high doses of viruses.

2.6.2.2 Adeno-associated viruses

Adeno-associated viruses are small (particle size 20nm), non-enveloped parvoviruses (Rabinowitz and Samulski, 2000). There are currently eight different human AAV serotypes, the most commonly used as gene therapy vector being AAV 2. Unless otherwise indicated, this paragraph focuses on AAV type 2.

AAV are able to infect both dividing and undividing cells, although they may have some preference to infect cells in S-phase. Wild type AAV integrate in a site specific manner to the chromosome 19q13.3, but with recombinant AAV site specific integration is lost and viruses integrate randomly or remain episomal. The cloning capacity of AAV is only about 4.5 kb but since AAV has a tendency to heterodimerize in head-to-tail orientation during concatamer formation, an expression cassette can be cloned into two different AAV vectors which are then co-transfected. Thus cloning capacity of AAV is almost 10 kb.

AAV has a 4.7 kb, linear single-stranded DNA genome that contains of two genes: *rep* codes replication and integration functions and *cap* codes structural components of the virus (Rabinowitz and Samulski, 2000). Structural proteins are translated from three overlapping transcripts of the cap gene (Vp1, Vp2 and Vp3) and regulatory proteins from four overlapping transcripts of the rep gene (rep78, rep68, rep52 and rep40). AAV has three promoters p5, p19 and p40. Transcripts initiating at these promoters have a common intron and they terminate at the same polyadenylation site. The only cis-acting components needed for recombinant AAV replication and packaging are two 145-nucleotide inverted terminal repeats (ITR). ITRs do not contain promoter/enhancer activity and therefore transgene expression is solely regulated by the elements inserted in the expression cassette.

AAV binds first to the heparan sulfate proteoglycan and uses $\alpha V\beta 5$ integrin and human fibroblast growth factor receptor 1 as co-receptors. AAV can transduce a large variety of cell types and at least retina, skeletal muscle, vascular smooth muscle cell and central nervous system cells have been reported to support recombinant transgene expression. The efficiency of AAV transduction depends on the serotype used. When AAV serotypes 1-5 were tested, AAV type 1 was efficient in liver and skeletal muscle transduction followed by types 5, 3, 2, and 4, whereas types 5 and 4 were more efficient in transducing retina (Rabinowitz et al., 2002). In addition, it has been shown that lung airway epithelium is resistant to transfection

with AAV2 but can be transduced with AAV6 opening possibilities to AAV mediated gene therapy of lung diseases such as cystic fibrosis (Halbert et al., 2001).

AAV are not associated with any human diseases and are considered nonpathogenic. Although AAV are considered to be rather non-immunogenic, between 50 to 96% of the population have antibodies against AAV type 2 and 18 to 68.5% have neutralizing antibodies. AAV transduction results in T-cell dependent activation of B-cells and production of neutralizing antibodies that block readministration of AAV. AAV capsid is mainly responsible of humoral reaction. Cytotoxic T-lymphocytes also eliminate AAV directly. AAV mediated transfection to carotid artery in New Zealand Rabbit is associated with modest T-cell infiltration in the intima, media and adventitia, that is absent from sham-operated control rabbits (Richter et al., 2000). AAV transfection also attracts few macrophages. It appears that administration of AAV intravenously causes more severe immunological reactions compared to intramuscular administration. Administration intravenously allows viruses to enter spleen which activates immunological cascades. Nevertheless, compared to adenoviruses AAV are much less immunogenic.

AAV expressing factor IX has been injected to the muscles of patients suffering from severe hemophilia B at the dose of 1.8×10^{12} vector genomes / kg. AAV vector sequences were detected in the serum, urine and saliva after gene transfer but not in semen. AAV were well tolerated and did not cause any major adverse effects. Neutralizing antibodies were detected in the serum before the gene transfer in 7 out of 8 patients and the level of antibodies rose after gene transfer. Muscle biopsies obtained 2, 6 or 10 months after gene transfer were positive for transgene analyzed by PCR and immunohistochemistry in 8 out of 10 biopsy samples. Four out of eight patients had increased factor IX levels compared to baseline and two patients were able to reduce the use of factor IX concentrate by 50%. However, circulating levels of factor IX were lower than what was required for therapeutic effect.

2.6.2.3 Retroviruses

Retroviruses are small RNA viruses and the most commonly used retroviruses are based on Moloney murine leukemia viruses (MMuLV). They infect cells through interaction between an envelope protein and a cell surface receptor protein in the target cell. Apart from lentiviruses, retroviruses can not infect permanently specialized, quiescent cells but require replication for entry into the nucleus. Since retroviruses integrate, they can produce a stable transgene expression but a major disadvantage, especially *in vivo* experiments, has been low titers (generally no higher than 10^6 cfu/ml), low transduction efficiency, transcriptional silencing of viral promoter limiting the duration of transgene expression and inability to infect undividing cells. On the other hand, in cancer gene therapy the retrovirus-ability to infect rapidly dividing tumor cells, and not normal, healthy cells, could be advantageous. Retroviruses may also be more useful in *ex vivo* gene transfer trials.

Many attempts have been made to improve retroviral vectors. Pseudotyping with naturally existing heterologous envelope proteins has been tried with much success. The most commonly used enveloped protein is vesicular stomatitis virus (VSV-G) envelope protein that broadens the host range of traditional MMuLV viruses and makes them more stable to endure concentrating. Concentrations as high as 10^{10} - 10^{11} cfu/ml have been reported.

Retroviral infection does not alter the growth properties of the host cell or cause severe immunological reactions. On the other hand, some malignancies are caused by retrovirus infection and insertional mutagenesis and germ line integration are possible. Indeed, it was recently found that two out of ten children with X-linked severe combined immunodeficiency

(SCID-X1) treated with retroviruses mediated gamma(c) gene developed T-cell leukemia three years after gene transfer. T-cell leukemia resulted from retrovirus integration in the proximity of the LMO2 proto-oncogene promoter, leading to aberrant transcription and expression of LMO2.

Replication competent retroviruses (RCR) replicate and multiply after initial transduction resulting in enhanced transfection efficiency (Weber et al., 2001). This approach raises much concern, for uncontrolled spread of replication competent viruses could lead to insertional mutagenesis and carcinogenesis. Indeed, three out of ten severely immunocompromised rhesus macaques developed fatal T-cell lymphomas after receiving bone marrow cells contaminated with RCR. Although similar experiment with less severe immunosuppression did not cause lymphoma in macaques, the result indicates that wider use of retroviruses in gene therapy trials could lead to adverse effects. The concern is hastened with recent recognition that human endogenous retroviruses make up approximately 1% of the human genome and recombination with endogenous retroviruses could result in neopathogens with the ability to deliver altered transgenes to undesired cell types. Endogenous retroviruses in porcine genome are transcriptionally active and capable of infecting human cells. It is possible, that after the leukemia cases with SCID-X1 patients RCR will not be used in human trials or - at the very least - a suicide gene or some other method enabling destruction of virus bearing cells is introduced in the vector.

2.6.2.4 Lentiviruses

Lentiviruses such as human immunodeficiency virus (HIV) belong to the family of retroviruses and have been used in gene transfer experiments *in vitro* and in animal models *in vivo*. Lentiviruses are integrating viruses and, unlike oncoretroviruses, they have a unique ability to transduce non-dividing cells enabling the gene therapy of terminally specialized cells such as neurons.

Like oncoretroviruses, lentiviruses contain *gag*, *pol* and *env* genes but in addition they have two regulatory proteins *Tat* and *Rev* and four accessory proteins *Nef*, *Vif*, *Vpu* and *Vpr* (Lever, 2000). However, six out of nine lentivirus genes can be deleted or expressed from a separate vector. In addition, the safety of lentivirus vectors was improved after introducing the self inactivating vector (SIN) (Miyoshi et al., 1998).

In addition to HIV other lentiviruses such as feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV) and equine infectious anemia virus (EIAV) have been tested (Vigna and Naldini, 2000). An envelope protein infectious to human cells has to be used with these vectors to enable transduction of human cells. The most commonly used envelope protein is VSV-G. However, if there are multiple steps limiting the transduction, the efficiency of the transduction may be too low for gene therapy purposes and if the viral entry is the only block hindering the transduction of human cells, non-primate lentiviruses may not differ from HIV lentiviruses in terms of biosafety. There is already evidence that FIV is able to transduce human peripheral blood mononuclear cells indicating the possibility of cross species infection by wild type viruses.

Since lentiviruses can cause a fatal disease their use in gene therapy purposes in human raises much concern (Vigna et al., 2002; Buchschacher and Wong-Staal, 2000). Formation of replication competent retroviruses (RCR) due to the homologous recombination within the producer cell lines could be possible. This concern is hastened by the fact that unlike in oncoretroviruses, lentiviral genes overlap increasing the risk of homologous recombination. As discussed above, the human genome contains endogenous retrovirus sequences (HERV)

that derive from ancient retrovirus infections. Recombination process between HIV based lentiviral vectors and HERV could lead to the production of RCR. On the other hand, the envelope protein of HERV could protect the host from retroviral superinfection by interfering with the receptor mediated uptake of retroviruses. If a patient first receives lentiviral gene therapy and then becomes infected with HIV could these two recombine and produce new kind of replication competent virus? As with all integrating viruses the risk of insertional mutagenesis can not be overlooked. In addition, lentiviral proteins are somewhat toxic to cells and thus multiplicity of infection (moi) has to be assessed carefully to avoid elimination of the transduced cells due to the toxic effect.

2.6.2.5 Baculoviruses

Baculoviruses are a group of species-specific insect viruses that are not known to replicate in mammalian cells (Pieroni and La Monica, 2001). They have long been used as bio-pesticides and are considered safe. Baculoviruses are not known to use a specific cell surface receptor for infection but use electrostatic interactions to bind mammalian cells, such as negatively charged moieties of heparan sulfate. If a mammalian promoter or a viral promoter able to function in mammalian cells (CMV, LTR) is used, baculoviruses drive strong gene expression. The same gene of interest under a baculovirus polyhedrin-gene promoter is not expressed, since the promoter is not active in a non-arthropod environment. This is also a safety issue, since baculovirus genes under baculovirus promoters can not be expressed in mammalian cells.

2.6.2.6 Herpes simplex viruses

HSV are able to infect several different cell types including liver and muscle and they infect both dividing and nondividing cells (Robbins et al., 1998). They can carry large DNA inserts and have a unique ability to establish latency in neuronal cells that could be advantageous in treating neurological diseases. A major obstacle of HSV vectors is the short transgene expression period. Gene expression is shut down within one week after infection.

2.6.3 Non-viral gene transfer methods

By comparison with viral gene transfer methods, non-viral techniques offer a relatively safe and cheap method to deliver foreign DNA to cells and allow repeated administration of gene(s). So far the low transfection efficiency and transient gene expression has limited their usefulness in *in vivo* trials (Li and Huang, 2000).

A simple gene transfer method is to deliver *naked DNA* by direct injection of plasmid into skeletal muscle or by introducing plasmid DNA through venous access (portal vein or tail vein) in a large volume. It has been reported that such intravenous gene transfer results in liver gene expression comparable to adenovirus mediated gene transfer (Liu et al., 1999).

Gene transfer of naked DNA can be facilitated by physical methods like electroporation or by gene gun. These approaches allow DNA to penetrate through the cell membrane and bypass endosomes and lysosomes. Using gene gun delivery DNA may enter straight into the nucleus. Gene gun delivery is especially suitable in gene transfer trials targeted to skin for skin is easily accessible. It has also been shown that some polymers may improve gene transfer efficiency possibly by protecting DNA against enzymatic degradation.

There are two types of liposomes, anionic and cationic of which cationic are more widely used in human trials. *Cationic lipids* complex with DNA forming aggregates of condensed DNA and lipid layers. Cationic liposomes have been widely used in gene transfer studies both *in vivo* and *in vitro* and based on these experiments it now appears that the route of administration greatly affects *in vivo* transfection efficiency and that there is discrepancy between *in vitro* and *in vivo* experiments. This is probably due to the physical properties of cationic lipid/DNA complexes. When charged complexes are infused to the circulation, they are subjected to serum which can cause aggregation, release of DNA from the complexes and DNA degradation. If the cationic lipid/DNA complex is infused straight into the tissues, complexes are less exposed to biological fluids. Cationic lipid/DNA complexes are also rapidly cleared by the reticuloendothelial system. Therefore different protocols should be designed for each clinical setting, making the cationic liposomes rather complicated in practice. Furthermore, the gene transfer efficiency with cationic liposomes is generally low.

DNA conjugates: DNA can also be conjugated to defective viral particles or virally derived peptides via linker molecules such as polylysine. The method utilizes the viruses' efficient ability to enter cells, disrupt the lysosome and/or transport DNA to nucleus. A downside is that the immunogenic properties of the viruses are also maintained.

RNA/DNA chimeras represent the early idea of gene therapy by repairing a mutated gene in the chromosome. An RNA/DNA chimera is designed so that it aligns perfectly with the genome. The chimera contains a pentameric DNA stretch with one base pair mismatch. The mismatch is recognized by the DNA repair system resulting in alteration of the DNA sequence of the targeted gene. It is not yet clear whether longer than one base pair mismatch could be corrected or whether deletion or insertion of one nucleotide could be accomplished by this method.

3 AIMS OF THE STUDY

The aim of this study was to create a soluble scavenger receptor (sMSR) and to test its function in inhibiting macrophage scavenger receptor mediated functions both *in vitro* and *in vivo*. In addition, the role of PAF-AH / Lp-PLA₂ on LDL oxidation was investigated. More specifically, the following questions were addressed:

1. Can a soluble macrophage scavenger receptor inhibit degradation of modified LDL *in vitro*? What is the effect of sMSR on degradation on MSR knock-out macrophages? Is it possible to inhibit foam cell formation *in vitro* by sMSR? (Study I)
2. Is it possible to inhibit lesion formation in LDLR knock-out mice by adenovirus mediated gene transfer of soluble macrophage scavenger receptor? What happens to the sMSR / modified LDL complex? (Study II)
3. What is the long term effect of AAV-mediated soluble macrophage scavenger receptor on atherosclerosis in LDLR knock-out mice? (Study III)
4. Can PAF-AH / Lp-PLA₂ protect LDL against oxidation and prevent foam cell formation *in vitro*?

4 METHODS

4.1 Production of gene transfer plasmids and viruses

Plasmids

The bovine growth hormone signal sequence, the extracellular parts of the human macrophage scavenger receptor (Kodama et al., 1990) and an eight amino acid Flag peptide (DYKDDDDK) were subcloned into a pcDNA 3 vector to create soluble macrophage scavenger receptor A I (figure 3A) (Article I).

A replication-deficient E1/E3 deleted adenovirus was generated, using a pAdvBgl plasmid (Barr et al., 1994). A human CD68 promoter, sMSR and SV40 polyA were subcloned into the 3' end of the first map unit of the adenovirus genome to create plasmid pCD68sMSRA-1.

To generate plasmid pCMVsMSRA-1, CMV promoter, sMSR and bovine growth hormone poly(A) were excised from pcDNA 3 vector and subcloned into the 3' end of the first map unit of the adenovirus genome.

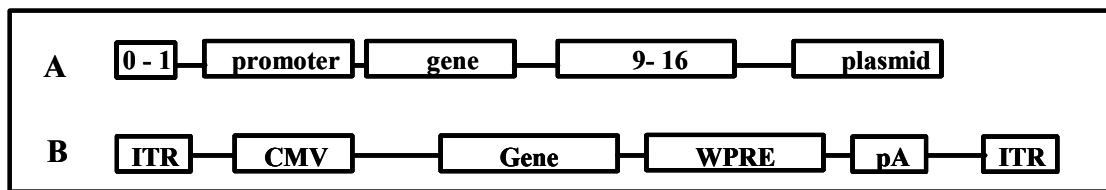


Figure 3: Adeno (A) and AAV (B) vectors.

Production of adenoviruses

Adenoviruses were produced in 293 cell line after co-transfection and homologous recombination of plasmid pCD68sMSRA-1 or pCMVsMSRA-1 and sub360 adenovirus genomic DNA.

PCR was used to confirm the presence of the transgene, with primers 5'- TAC AAG GAC GAC GAT GAC -3' (upper primer) and 5'-GTA AAC ACG CTC CTC TAA -3' (lower primer) annealing to the Flag epitope and sMSR, respectively. Viruses were analyzed for toxicity and for the absence of wild-type viruses (Horwitz, 1996) and tested for the absence of microbiological contaminants, mycoplasma and lipopolysaccharide using standard methods (Laitinen et al., 1998).

The PAF-AH adenoviruses were constructed and produced with the Adeno-XTM Expression System (figure 3A). Human PAF-AH cDNA was cloned into Adeno-X-Viral DNA with CMV immediate early promoter and bovine growth hormone polyA. Fugene 6 was used for plasmid transfection and viruses were produced in HEK 293 cell line.

Production of recombinant adeno-associated viruses (rAAV)

AAV vector psub-CMV-sMSR-flag-WPRE (AAVsMSR) encoding sMSR under the CMV promoter and Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) was generated (figure 3B) (Paterna et al., 2000). Adenovirus helper plasmid, pBS-E2A-VA-E4, as well as AAV packaging plasmid, pAAV-/Ad-rep(ACG), were used in production of the rAAV (Paterna et al., 2000) and purified in iodixanol gradient (Zolotukhin et al., 1999). The recombinant virus was purified by heparan sulfate sepharose HPLC. The titer of the virus was analyzed by slot blot. EGFP virus was generated as described (Paterna et al., 2000).

4.2 Cell culture

In vitro adenovirus transduction

RAW 264.7 macrophages, NIH3T3, ECV 304 or RAASMC (Ylä-Herttuala et al., 1995) cells were transduced with recombinant adenoviruses at a multiplicity of infection of 100-1000-5000. Medium was replaced with Optimem media containing 10% or 0.5% (v/v) LPDS and collected at 12-hr intervals. Some samples were lyophilized.

Southern and Northern blots

Southern blot: Adenovirus genomic DNA was isolated and restriction enzyme digested. DNA was subjected to electrophoresis, transferred to a nylon membrane and hybridized with a random-primed DNA probe specific for both native and secreted scavenger receptors. Autoradiography was used for signal detection (Ausubel F et al., 1995).

Northern blot: Cells were transduced with sMSR or LacZ adenoviruses. mRNA was isolated with oligo-dT cellulose resin, subjected to electrophoresis, transferred to a nylon membrane and hybridized with a random-

primed probe specific for the sMSR. The membrane was stripped and reprobed with a human β -actin probe (Ausubel F et al., 1995).

Ligand binding assay

sMSR containing media or control media was mixed with poly(G)-conjugated resin. The mixture was centrifuged and the supernatant, containing trace amounts of sMSR, was mixed with the Western blot sample buffer. The resin pellet, containing the bound sMSR, was mixed with the Western blot sample buffer and sMSR was released from the resin by boiling (Resnick et al., 1993).

Western blot

Samples from ligand binding assay or conditioned media from transduced cells was subjected to standard SDS polyacrylamide gel electrophoresis with 4% (w/v) stacking gel and 8% (w/v) (sMSR) or 12% (PAF-AH) separating gel under reducing or non-reducing conditions. Protein was transferred to a nitrocellulose (sMSR) or PVDF membrane (PAF-AH). Expressed protein was detected with a mouse anti-Flag monoclonal antibody M2 (sMSR) or anti-human PAF-AH polyclonal antiserum, biotinylated horse anti-mouse IgG secondary antibody and streptavidin-alkaline phosphatase using BCIP-NBT as substrate.

Dot blot assay

The amount of sMSR protein in the transfection medium was measured using a dot blot assay. Dilutions of the transfection medium were spotted on a nitrocellulose membrane along with standards ranging from 0.01ng to 200ng/100ul. Proteins were detected with anti-Flag monoclonal antibody M2, biotinylated horse anti-mouse IgG secondary antibody and streptavidin-alkaline phosphatase using BCIP-NBT as substrate and quantified using an image analyzer and MCID-M4 program (Imaging Research Inc., Brock University, St. Catharine, Ontario, Canada).

Isolation and modification of LDL

LDL was isolated from fasting plasma of healthy normolipidemic donors by sequential ultracentrifugation (Havel et al., 1955) and radioiodinated (Bilheimer et al., 1972) before acetylation (Goldstein et al., 1979) and oxidation (Ylä-Herttuala et al., 1989). For PAF-AH experiments LDL was isolated from PAF-AH transduced rabbits, radioiodinated and oxidized. A portion of LDL was treated with PAF-AH inhibitor. Specific activities of the labeled LDLs were 250-800 cpm/ng protein.

Degradation assay

RAW 264 macrophages or peritoneal macrophages from MSR AI/II knock-out mice or from LDLR / MSR AI/II double knock-out mice were incubated with 125 I-AcLDL or 125 I-oxLDL and conditioned medium containing sMSR. Alternatively cells were incubated with 125 I-oxLDL that had been isolated from PAF-AH transduced rabbits. After precipitation of free 125 Iodine and non-degraded 125 I-acetylated LDL / 125 I-oxidized LDL the trichloroacetic acid-soluble noniodide radioactivity was measured from the media and cells with γ -counter and results were corrected for protein concentration (Henriksen et al., 1981; Steinbrecher et al., 1984; Ylä-Herttuala et al., 1989).

Foam cell formation

RAW 264 cells were incubated with conditioned media containing sMSR, 100 μ g/ml of AcLDL and 4 μ g/ml of phagocytosis inhibitor cytochalasin D or with Optimem containing OxLDL that had been isolated from PAF-AH transduced rabbits for 18-24h. After the incubation the cells were, fixed with 4% paraformaldehyde and stained with Oil Red O and hematoxylin (Ylitalo et al., 1999).

Enzyme-linked immunosorbent assay (ELISA)

sMSR was detected from conditioned media or from mouse/rabbit plasma with ELISA with polyclonal rabbit anti flag M2 antibody and polyclonal goat anti scavenger receptor antibody. HRP-conjugated anti goat antibody and peroxidase substrate (3,3',5,5'- Tetramethylbenzidine) were used for detection of the protein. Absorbances were measured at 450nm with Elisa reader Multiscan RC (ThermoLabsystems, Helsinki, Finland) and compared to the standard curve.

Adhesion test

EA.hy 926 cells were grown with OxLDL for 24h. THP-1 cells or PMA activated THP-1 cells were labeled with calcein and incubated with conditioned medium containing sMSR or with control LacZ medium. 60 000-

100 000 cells were plated on the activated EA.hy 926. Fluorescent activity of the adherent cells was measured with fluorometer (Perkin Elmer HTS 7000 Plus Bio Assay Reader, MA, USA).

PAF-AH activity analysis

Plasma and LDL PAF-AH activity was determined using a commercially available assay kit based on hydrolysis of 2-thio-PAF by PAF-AH. Free thiols were detected using 5,5'-dithiobis-2-nitrobenzoic acid (Ellman's reagent) and the absorbance was read at 414nm by an ELISA reader. Absorbance values were plotted as a function of time and the PAF-AH was calculated from the linear portion of the curve and expressed as $\text{nmol}\cdot\text{ml}^{-1}\cdot\text{min}^{-1}$.

4.3 In vivo techniques

New Zealand White rabbit

NZW rabbits were fed a 0.5% cholesterol diet for two weeks. Food and water were provided ad libitum. Animals were anesthetized using fentanyl-fluanisone (0.3ml/kg s.c.) and midazolam (1.5 mg/kg i.m.). 10^8 , 10^9 or 10^{10} pfu of recombinant PAF-AH or LacZ adenoviruses were injected to the carotid artery via a 5.0 F introducer. Seven days later plasma was collected and the rabbits were sacrificed. All animal experiments were conducted in accordance with guidelines of the Animal Protocol Review Committee of Kuopio University.

LDLR knock-out mouse

LDLR knock-out mice (Ishibashi et al., 1994) or LDLR/MSR A I / II double knock-out mice were used in the studies. At the age of 2 months Western-type diet containing 21% fat (wt/vol), 0.15% cholesterol (wt/wt) and 19.5% casein (wt/wt) without sodium cholate was started (Plump et al., 1992). Diet and water were provided ad libitum. Animals were anesthetized using fentanyl-fluanisone (3.15 mg/kg and 10mg/kg) / midazolam (5mg/kg). Blood samples were taken from tail vein with heparinized capillaries. After the experiment animals were sacrificed using carbon dioxide.

For gene transfer experiments mice were divided in four groups (figure 4). The first group of mice was kept on Western type diet for ten weeks, gene transferred with CD68sMSR adenoviruses and sacrificed six weeks later. The second group was kept on Western type diet for 6 weeks, gene transferred with CMVsMSR adenoviruses and sacrificed four weeks later. The third group was like the first group but CMVsMSR adenoviruses were used. With the fourth group diet was started at the time of the gene transfer with AAVsMSR and mice were sacrificed six months later. Mice were transfected with a single tail vein injection of recombinant viruses (10^9 pfu of adenoviruses or 7.5×10^9 particles of adeno-associated viruses). LacZ adenoviruses or EGFP AAV were used as controls. Blood samples were collected at various points of the trail.

In the organ distribution of sMSR-AcLDL complexes, conditioned media with or without sMSR protein was combined with ^{125}I -AcLDL and injected in the tail vein of anesthetized LDLR/MSR AI/II double knock out mice. Mice were sacrificed 30 minutes later and organs were analyzed for radioactivity with gamma counter.

In the turnover study LDLR knock-out mice were injected with recombinant adenoviruses as indicated above. Three days after the gene transfer mice were tail vein injected with $100\mu\text{g}$ of ^{125}I -AcLDL or ^{125}I -OxLDL. At indicated time points mice were bled from the tail vein and serum samples were analyzed with gamma counter.

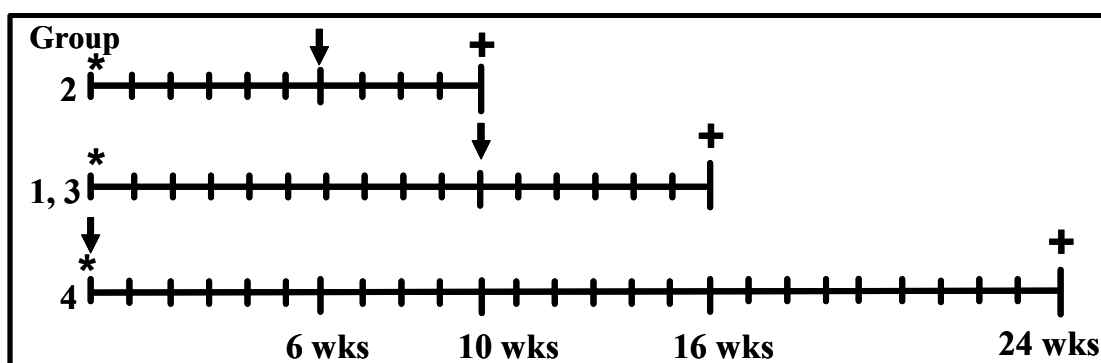


Figure 4: Time table of *in vivo* experiments with LDLR knock-out mice transduced with viruses expressing sMSR or control LacZ/EGFP. Group 1: AdCD68sMSR; group 2-3: AdCMVsMSR and group 4: AAVsMSR. * beginning of Western type diet, ↓ gene transfer, + sacrifice.

Analysis of atherosclerotic lesion area and volume in LDLR knock-out mice

Mice were sacrificed using carbon dioxide and perfused with PBS and 4% paraformaldehyde through a small hole in the right atrium, fixed for 2 hr in the same fixative and immersed in PBS over night (Ylä-Herttuala et al., 1990). Fixed aortas were opened and pinned out under dissection microscope (Leppänen et al., 1998). Aortic lesions were detected and lesion areas were measured with an image analyzer and MCID-M4 program (Imaging Research Inc., Ontario, Canada). Lesion area from each aorta was measured in a blinded fashion three times and average value was reported. After the analysis open aortas were embedded in paraffin and 7µm sections were cut. Lesion volumes were analyzed from sections after immunohistochemical or hematoxylin-eosin staining at standardized points on valve level using Image-Pro Plus 3.0 program (Media Cybernetics, MD, USA).

Immunohistochemistry

Rabbit samples were fixed in paraformaldehyde and immersed in PBS. Mice were perfused with PBS and paraformaldehyde, fixed for 30 min (x-gal staining) or 2 h (immunohistochemistry) in the same fixative and immersed in PBS over night (Ylä-Herttuala et al., 1990). Tissues were embedded in paraffin and/or in OCT compound and 7µm and 10µm sections were cut from the block, respectively. Macrophages (mMQ; 1:5000) and oxidized epitopes (Mal-2 (Palinski et al., 1989); 1:100) were detected from the mouse aortic sections. Anti-flag monoclonal antibody (M2; 10µg/ml) was used to detect flag epitope in sMSR in liver sections. Anti-human PAF-AH antiserum was used to detect PAF-AH from rabbit liver sections.

PCR and RT-PCR

Total RNA and DNA were isolated with TRIzol compound. RNA was treated with DNase. cDNA was synthesized from the total RNA with M-MuLV Reverse Transcriptase and random primers. Amplification of the transgene was performed from cDNA samples and also from DNase treated RNA samples for the detection of possible genomic DNA contaminants. Amplification primers for sMSR were 5'-TACAAGGACGACGATGAC-3' and 5'-CCAGTGGGACCTCGATCTCC-3' annealing to the Flag epitope and MSR, respectively. Hot start (95°C 5 min) was followed by 40 cycles (95°C 45 s, 57°C 30 s, 72°C, 60s) with the final extension of 10 min at 72°C. 2µl of the first PCR product was taken to the second PCR reaction with primers 5'-GGACGACGATGACAAGGCGG-3' and 5'-TTGCATTCCCATGTCCCTGG-3' both annealing to the MSR (95°C 3 min and 40 cycles of 95°C 45 s, 72°C 90 s, with the final amplification of 10 min at 72°C). Amplification primers for human PAF-AH were 5'-TGGAGCAACGGTTATTCAG-3' and 5'-TGGTTGTGTTAATGTTGGTCC-3' (45 cycles of 94°C 60s, 62°C 60s, 72°C 60s). Amplification primers for LacZ PCR were 5'-TTGGAGGCCTAGGCTTTTGC-3' and 5'-ATACTGTCGTCGTCCTCCCTCA-3' (95°C 5 min, 80°C 3 min and 40 cycles of 95°C 45 s, 58°C 45 s, 72 °C 50 s with the final extension of 5 min at 72°C). 2µl of the first PCR product was taken to the second PCR reaction with primers 5'-GGTAGAAGACCCCAAGGACTTT-3' and 5'-CGCCATTCGCCATTCAG-3' and 40 cycles (95°C 60s, 58°C 60s, 72°C 60s) with the final extension at 72°C for 5 min.

Statistical analyses

Independent samples t-test or ANOVA was used to evaluate statistical significance. $P < 0.05$ was considered statistically significant. Numerical values for each measurement are shown as mean \pm SD or \pm SEM.

5 RESULTS

5.1 Characterization of CD68-sMSR viruses and effect on foam cell formation (J)

To study the effect of sMSR, various recombinant viruses were produced. First, sMSR was subcloned into an adenovirus vector under the macrophage specific CD68 promoter. The correct structure of CD68sMSR construct was verified by sequencing viral genomic DNA and by Southern blot. The correct sized messenger RNA (1657bp) was detected in Northern blot after adenovirus-mediated transfection. Under the CD68 promoter sMSR was produced in RAW 264.7 macrophages but also in ECV 304 endothelial cells. NIH 3T3 fibroblasts and RAASMC produced only trace amounts of sMSR. Most of the protein was monomeric but also dimeric protein was detected. However, trimeric protein was not detected by Western blot. Pre-incubation of sMSR with poly(G) resin reduced the amount of sMSR in Western blot almost entirely, indicating that sMSR could bind MSR ligand. Conditioned sMSR media produced in RAW 264.7 or ECV 304 cells during 12 h incubation contained 0.1-2.5ng/ μ l of sMSR protein when tested by quantitative dot blot assay. sMSR produced in NIH 3T3 and RAASMC was below the detection level of the assay. Based on the quantitative dot blot assay it was calculated that the conditioned medium used for the competition studies contained 2.5 μ g/ml sMSR protein vs. 10 μ g/ml of acetylated LDL. If assuming a molecular weight of 2.5 $\times 10^6$ Da for acetylated LDL and 135 kDa for dimeric sMSR and 200kDa for trimeric sMSR the conditioned medium contained approximately five dimeric sMSR molecules or three trimeric sMSR particles for every LDL particle. The predicted structures of the hMSR AI and sMSR are illustrated in figure 5.

We next demonstrated the functionality of sMSR protein. sMSR that had been produced in RAW 264.7 cell line after adenovirus mediated transduction inhibited the degradation of 125 I-AcLDL and 125 I-OxLDL by 70% and 80%, respectively (Figure 6A). Cumulative inhibitory effect was detected when both sMSR media and unlabelled modified LDL was used in the

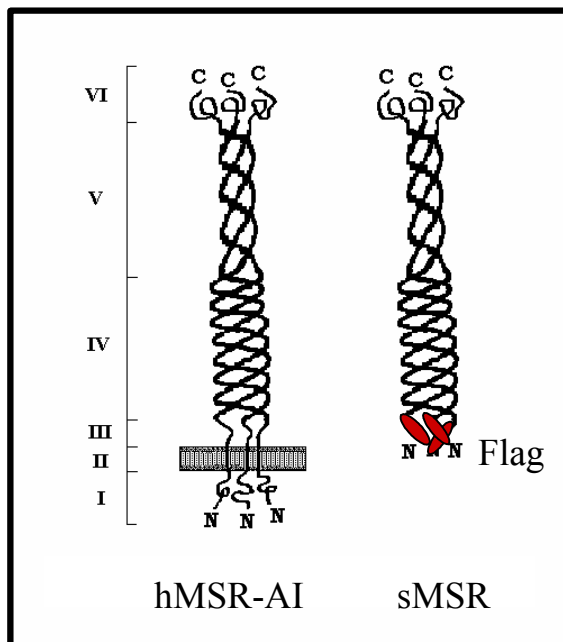


Figure 5: Illustration of the hMSR AI and the predicted structure of the sMSR. I: cytoplasmic domain, II: Transmembrane domain, III: Spacer domain, IV: α -helical coiled coil domain, V: collagen like domain, VI: C-terminal cysteine rich domain. Picture drawn by Riina Innilä.

degradation assay. sMSR also inhibited the degradation of ^{125}I -AcLDL in MSR A knock-out cells by 70%. However, pre-incubation of sMSR media with macrophages did not affect degradation. In a foam cell formation assay with $100\mu\text{g/ml}$ of AcLDL and $4\mu\text{g/ml}$ of cytochalasin D sMSR was able to inhibit lipid uptake and foam cell formation by RAW 264.7 macrophages.

When injected *in vivo* sMSR – ^{125}I -AcLDL complexes were accumulated in the liver.

5.2 Expression of sMSR *in vivo* (II)

We next studied whether sMSR can affect atherosclerosis *in vivo*. After a tail vein gene transfer of CD68sMSR (Figure 4, group 1) expression was detected frequently in liver but also in spleen, kidney and lung by genomic PCR and RT-PCR (Table 5). sMSR protein was detected in the liver of transfected mice by anti-flag monoclonal antibody. CD68sMSR mice had 7-12% less atherosclerotic lesions in the aorta compared to LacZ controls – a result that was not included in the article II.

Because of the relatively weak antiatherogenic result obtained with CD68sMSR adenoviruses, sMSR was subcloned under the control of a universal CMV promoter. The correct structure of the CMVsMSR adenoviruses was verified by sequencing viral genomic DNA. Conditioned media from RAASMC after adenovirus-mediated transfection contained an average of $25\mu\text{g/ml}$ of sMSR (700ng/ml - $60\mu\text{g/ml}$) per 12 h collection, when analyzed by a sensitive ELISA. In Western blot monomeric and dimeric protein was detected. The size of the monomeric protein produced in ECV 304, NIH 3T3 and RAASMC was slightly smaller than the protein produced in RAW 264.7 macrophages (data not shown). In the degradation assay sMSR that had been produced in RAASMC inhibited the degradation of AcLDL and OxLDL by 70-80% and 30-50%, respectively (unpublished results).

In vitro incubation of THP-1 monocyte / macrophages with conditioned sMSR medium decreased the macrophage adhesion to OxLDL activated EA.hy 926 endothelial cells by 54% (Figure 6B and III).

Gene transfer of AdCMVsMSR via tail vein resulted in the production of sMSR mRNA and protein. Three days after the gene transfer sMSR expression was detected frequently in liver,

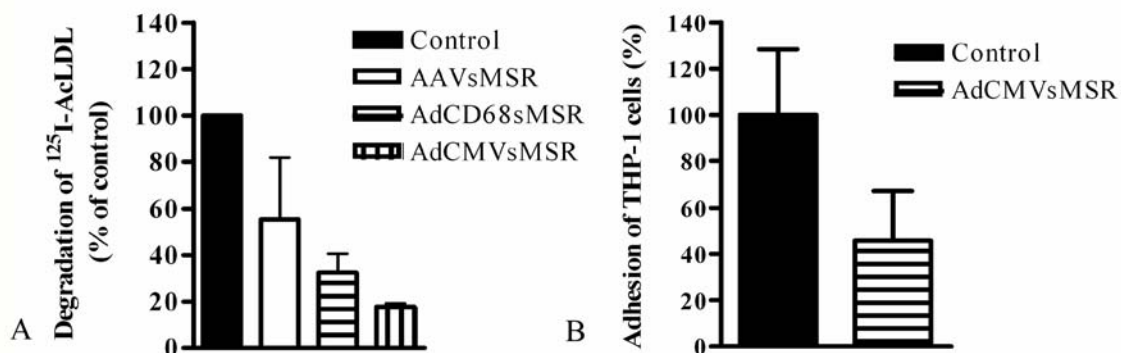


Figure 6: A) Degradation of ^{125}I -AcLDL in RAW 264.7 cells after AdCD68sMSR, AdCMVsMSR or AAVCMVsMSR gene transfer compared to control. B) Effect of sMSR or control LacZ incubation on THP-1 macrophage adhesion on OxLDL activated EA.hy926 endothelial cells.

spleen and suprarenal gland by genomic PCR and RT-PCR. Expression was also detected in kidney, lung, heart, and aorta, but there was some individual variation between mice (Table 5). With RT-PCR sMSR was found in liver as long as four weeks after the gene transfer. sMSR protein was detected in liver sections and also in the circulation by a sensitive ELISA. There was an average of 232 ng/ml of sMSR (89-349ng/ml) in the serum three days after the gene transfer. If the amount of virus was doubled, there was over 1 μ g/ml of sMSR in the serum four days after the gene transfer. Seven days after the gene transfer the amount of protein had declined and there was an average of 108 ng/ml of sMSR (79-142 ng/ml). sMSR was detected in the serum as long as four weeks after the gene transfer (average 16ng/ml) by ELISA (Figure 7).

No difference was seen in serum total cholesterol or triglyceride levels between sMSR and control LacZ groups after 16weeks of diet (Figure 4, group 3) (cholesterol baseline before gene transfer 16.75 ± 5.39 mmol/l in LacZ group vs. 20.02 ± 5.90 mmol/l in the sMSR group, cholesterol at 16 wks 23.54 ± 11.42 mmol/l in the LacZ group vs. 22.95 ± 9.7 mmol/l in the sMSR group). There were also no differences in total cholesterol or triglyceride levels between the groups after 10 week diet (Figure 4, group 2) (data not shown).

Turnover of 100 μ g of 125 I-AcLDL was analyzed three days after the gene transfer with CMVsMSR and CD68sMSR adenoviruses. In mice expressing CMVsMSR the clearance of 125 I-AcLDL was delayed by 20% at 2 min and by 50% at 10 min as compared to the LacZ expressing control mice (figure 8A). In CD68sMSR expressing mice the clearance of AcLDL was delayed by 13% and 24% at two and ten minutes respectively (Figure 8A, unpublished observation). Studies with 125 I-OxLDL gave similar results (Figure 8B, unpublished result).

Liver sections from mice transduced either with CMVsMSR or control LacZ adenovirus were analyzed with apoptosis kit ApoTaq. No difference was detected in the rate of apoptosis between sMSR and control sections (data not shown).

sMSR reduced lesion area in LDLR knock-out mice aortas. In the group 2 where the gene transfer was done at the time of the appearance of the first fatty streak lesions (Ishibashi et al., 1994) sMSR inhibited en face aortic atherosclerosis by 14% ($p < 0.05$). In group 3, where the gene transfer was done after the formation of the first fatty streaks, sMSR inhibited

Tissue	CD68sMSR RT-PCR	CD68sMSR GenPCR	CMVsMSR/LacZ RT-PCR	CMVsMSR genPCR	AAV gen-sMSR	AAV RT-PCR
Skeletal muscle	ND	ND	ND	ND	0/5	ND
Epididymis	ND	ND	ND	ND	0/5	ND
Testis	ND	ND	ND	ND	2/5	ND
Kidney	1/5	3/4	4/5	3/3	1/5	ND
Lung	0/5	3/4	4/5	3/3	0/5	ND
Heart	ND	ND	3/5	0/3	0/5	ND
Adrenal gland	ND	ND	5/5	2/3	3/5	ND
Spleen	2/5	4/4	5/5	3/3	4/5	ND
Liver	2/5	4/4	5/5	3/3	4/5	ND
Aorta	ND	ND	2/5	2/3	ND	3/11*

Table 5: Transgene expression *in vivo*. *Aortic samples from mice one (n=5), two (n=3) or three (n=3) months after gene transfer were examined. All three aortas three months after gene transfer were positive for the transgene. ND=not determined.

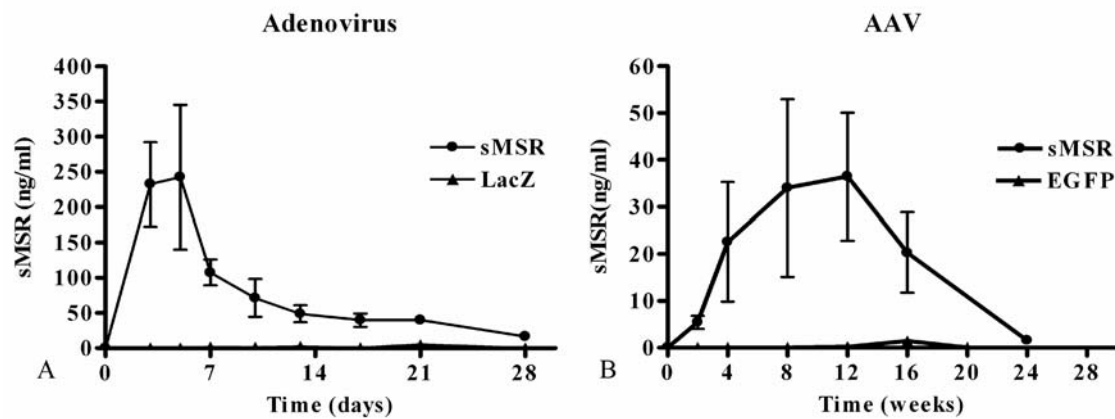


Figure 7: Serum concentration of sMSR after adenovirus mediated (A) or AAV mediated (B) gene transfer.

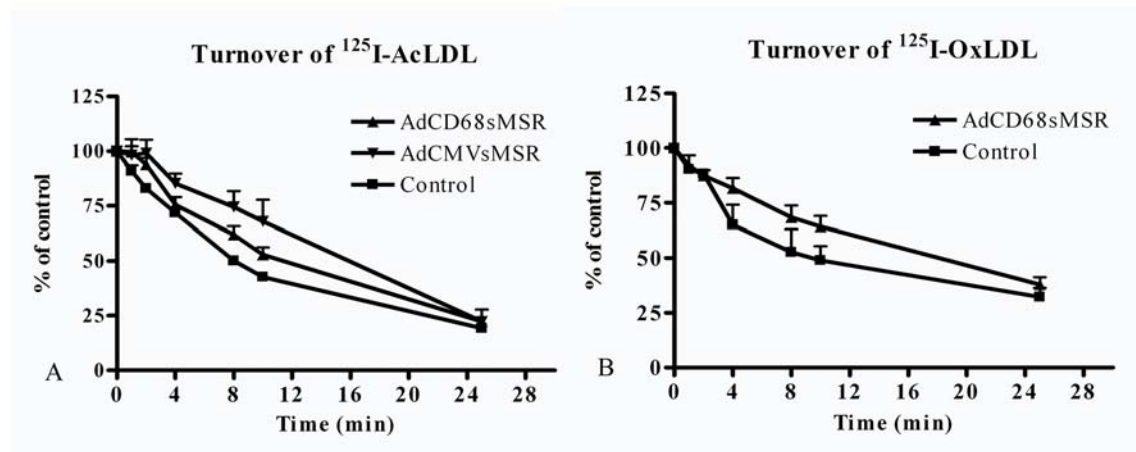


Figure 8: Turnover of ^{125}I -AcLDL after AdCD68sMSR, AdCMVsMSR or AdCMVLacZ transduction (A) and turnover of ^{125}I -OxLDL after AdCD68sMSR or AdCMVLacZ transduction (B) in LDLR knock-out mice. The results regarding ^{125}I -AcLDL and AdCMVsMSR viruses have been published (II).

atherosclerosis by 19% ($p=0.01$) (Figure 9, Table 6). There was no effect on the cellular composition of the lesions or the lesion volume in the valve level as analyzed from histological sections.

5.3 Characterization and *in vivo* expression of AAVsMSR (III)

In order to study the long term effect, sMSR was cloned into an AAV vector containing WPRE, under the CMV promoter. The correct structure of the plasmid was verified by sequencing the ligation junctions.

Conditioned media collected from WHHL rabbit fibroblasts 2-8 days after AAVsMSR transfection contained an average of $5\mu\text{g/ml}$ of sMSR (180ng/ml to $15\mu\text{g/ml}$, MOI 75) per 24h collection and conditioned media from RAASMC after AAVsMSR transduction contained an average of 2.5ng/ml of sMSR ($1\text{-}3.6\text{ng/ml}$) per 24 h collection. Protein was

detected in WHHL fibroblasts, RAASMC and HeLa cells after transduction by immunocytochemistry (data not shown). In the degradation assay conditioned media from WHHL fibroblasts inhibited the degradation of AcLDL by 35-84%.

After the systemic gene transfer sMSR genomic DNA was detected in the liver, spleen and suprarenal gland but also in heart, kidney and testis (table 5). RT-PCR from aortic samples showed gene expression three months after the gene transfer (3 out of 3 mice) but not one or two months after the gene transfer (0 out of 8 mice). AAVsMSR gene transfer lead to the transgene expression that could be detected by ELISA two weeks after the gene transfer (5.4ng/ml) and that peaked two to three months after the gene transfer (34.0-36.5ng/ml).

The gene transfer caused a temporary peak in the level of AST two weeks after the gene transfer. AST returned back to the baseline eight weeks after the gene transfer. There was no change in CRP after the gene transfer.

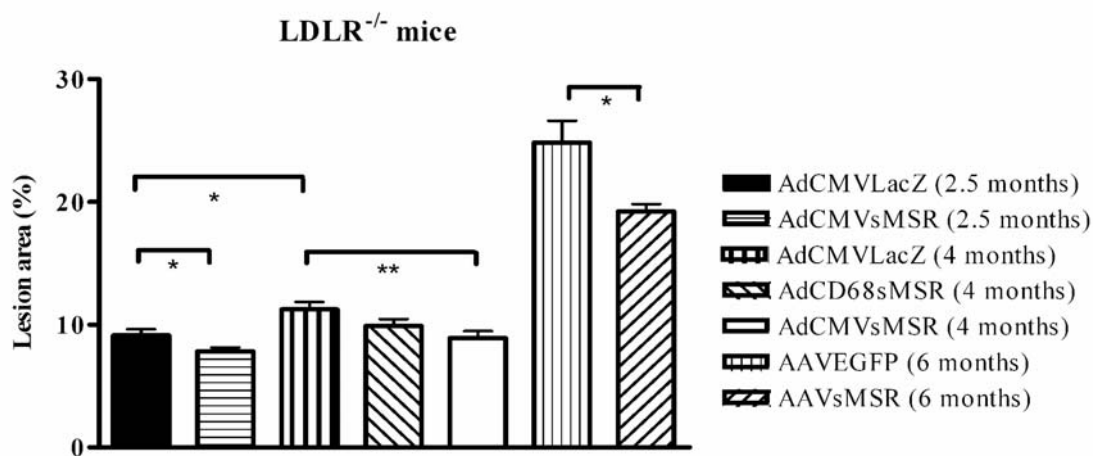


Figure 9: Effect of sMSR gene transfer on aortic atherosclerosis with different viruses and lengths of Western type diet. Data composed of different sets of independent experiments. * p<0.05; ** p≤0.01

Group	Diet before gene transfer	Sacrifice	Virus	Titer	Lesion reduction
1	10 weeks	16 weeks / 4 months	AdCD68sMSR	10 ⁹ pfu	7-12%; ND
2	6 weeks	10 weeks / 2.5 months	AdCMVsMSR	10 ⁹ pfu	14%; p<0.05
3	10weeks	16 weeks / 4 months	AdCMVsMSR	10 ⁹ pfu	19%; p=0.01
4	0 weeks	24 weeks / 6 months	AAVCMVsMSR	7.5x10 ⁹ vp	21%; p<0.05

Table 6: Effect of sMSR gene transfer on aortic atherosclerosis in LDLR knock-out mice with different sMSR expressing viruses and titers. Atherosclerotic lesion area is measured as percentage of the whole aortic area with an image analyzer and MCID-M4 program.

sMSR reduced lesion area in LDLR knock-out mice aortas by 21% ($p < 0.05$) six months after the gene transfer [$23.7\% \pm 1.9$ (EGFP, $n=12$), $19.2\% \pm 0.6$ (sMSR, $n=12$)] (Figure 9, Table 6). Atherosclerotic lesions were rich in macrophage-derived foam cells. There were no statistically significant differences in the lesion volume or the complexity of the lesions as analyzed from histological sections taken from the aortic valve level. Also, no significant differences were found in plasma cholesterol and triglyceride levels during the study.

5.4 Characterization and *in vivo* expression of PAF-AH adenoviruses (IV)

In order to study the effect of PAF-AH overexpression on LDL oxidation and atherogenesis related functions, PAF-AH expressing adenoviruses were generated. The correct structure of the PAF-AH adenoviruses was verified by sequencing and also by Western blot. Correct size protein (65kDa) corresponding to the known glycosylated size of a human PAF-AH was detected in Western blot. PAF-AH enzyme activity analysis showed over 10-fold increase in PAF-AH activity in conditioned medium collected from transduced SMC and RAW 264.7 compared to LacZ transduced control medium.

RT-PCR from transduced rabbit livers seven days after the gene transfer revealed human PAF-AH expression in the PAF-AH transduced animals but not in the control LacZ livers. Gene transduction of PAF-AH lead to the dose dependent increase in the rabbit serum PAF-AH activity: at an adenovirus dose of 10^8 pfu the increase was 7nmol/min/ml, at a dose of 10^9 pfu 13nmol/min/ml and at a dose of 10^{10} pfu the increase was 25nmol/min/ml compared to LacZ controls. Human PAF-AH protein was detected in the liver by immunohistochemistry but not in the liver of LacZ transduced rabbit.

Agarose gel electrophoresis showed a decrease in the electrophoretic mobility of the *in vitro* oxidized LDL sample from the PAF-AH transduced rabbit compared to LacZ control and also compared to LDL samples where PAF-AH activity was irreversibly inhibited with Pefabloc.

LDL was isolated from rabbits transduced with 10^{10} pfu of PAF-AH or LacZ adenoviruses, subjected to oxidation and used in the degradation assay with RAW 264. Increased PAF-AH content in LDL particles decreased the degradation of LDL after oxidation by 13-40% compared to LacZ transduced rabbits. The inhibition varied at different time points and was 13% at 3h, 40% at 6h, 23% at 12h and 26% at 24h. The effect was also dependent on the original amount of PAF-AH-adenovirus used for gene transfer and varied between 13-37%. Inhibition in the degradation was greatest with the highest level of PAF-AH activity. Inhibition of PAF-AH lead to 2-fold increase in the degradation compared to LacZ control. Similar results were obtained with human LDL.

Next we studied the effect of PAF-AH in foam cell formation assay. 100 μ g/ml of LDL isolated from transduced rabbits was oxidized and incubated with murine macrophages for 18h. Lipid accumulation and foam cell formation was inhibited with OxLDL containing the highest PAF-AH activity. Inhibition of PAF-AH led to an increase in lipid accumulation and foam-cell formation compared to non-inhibited PAF-AH LDL and LacZ LDL groups (IV).

6 DISCUSSION

Anti-ischemic and antithrombotic drugs as well as PTCA, stenting and by-pass surgery are effective in the treatment of atherosclerosis. However, development of DNA technology has made gene therapy a promising treatment option of the future.

sMSR *in vitro* (I-III)

MSR A plays an important role in atherogenesis by promoting lipid accumulation and foam cell formation in macrophages (Kodama et al., 1990; Rohrer et al., 1990) (Brown and Goldstein, 1983; Krieger, 1997; Greaves et al., 1998a). MSR A is also an adhesion molecule, a receptor for apoptotic cells (Platt et al., 1996; Terpstra et al., 1997) and it has a role in host defense (Greaves et al., 1998a). Experiments performed with MSR AI/II knock-out mouse models have proven that although foam cell formation can not be completely eradicated, the absence of MSR AI/II clearly inhibits atherosclerosis (Sakaguchi et al., 1998; Babaev et al., 2000; Suzuki et al., 1997).

Many transmembrane receptors have soluble counterparts to regulate their functions. They are interesting tool in gene therapy because they increase the efficiency of gene transfer (Park et al., 1998; Taguchi et al., 2000) (Quinn et al., 1997; Lenzlinger et al., 2002; Wajih et al., 2002). Soluble molecules spread the therapeutic effect to neighbouring cells and the demand for more efficient vectors can be somewhat circumvented.

We have used soluble receptor approach to inhibit modified LDL degradation, foam cell formation and monocyte/macrophage adhesion mediated by the MSRs in order to limit atherosclerosis in a hypercholesterolemic mouse model. sMSR was cloned under the tissue specific human macrophage CD68 promoter which directs high expression level in macrophages (Jones et al., 1998; Greaves et al., 1998b). A macrophage specific promoter was chosen because in human atherosclerotic lesions most of the scavenger receptor activity is expressed by macrophages (Ylä-Herttuala et al., 1991; Naito et al., 1992; Gough et al., 1999) and because transcriptional targeting of the transgene is an additional safety feature. However, since the 2.9 kb CD68 promoter used here did not contain the complete macrophage locus control region, endothelial cells could express sMSR as well. However, the expression in endothelial cells was considered beneficial in terms of possible local *in vivo* applications in the treatment of atherosclerosis and possibly restenosis. Endothelial cells can oxidize LDL and they are involved in monocyte recruitment to atherosclerotic lesions. The level of gene expression in fibroblasts and smooth muscle cells was low. Thus, the CD68sMSR adenovirus was able to direct clearly detectable transcriptional targeting to cell types involved in the processing of modified LDL in the arterial wall.

When the virally encoded sMSR was expressed in RAW 264.7 cells monomeric and dimeric sMSR protein was detected in Western blot but not trimeric protein which is believed to be the functional form of the MSR A I (although there are results indicating that also monomer could be active in ligand binding (Via et al., 1992)). MSR dimer is formed by strong disulfide bonds between two monomers but the third monomer is attached to dimer by weak, non-covalent forces. This may have caused the detachment of the third monomer from the trimer during the sample handling for SDS-page. Poly(G) resin was able to bind almost all of the sMSR in the medium. This indicates that although the trimer was not detected there must have been sMSR trimers in the conditioned medium. Similar results with poly(G) resin has previously been reported by others (Resnick et al., 1993) and it was concluded that sMSR presents similar ligand binding characteristics as the native MSR.

sMSR adenoviruses under the CMV promoter were also analyzed in Western blot. In addition to the monomer and dimer, a third fragment slightly smaller than the normal monomer was detected in ECV 304 endothelial cells, NIH3T3 fibroblasts and RAASMC but not in RAW 264.7 cells after AdCMVsMSR transduction (data not shown). The smaller fragment is most likely an anglycosylated / less glycosylated form of sMSR. The smaller fragment was not present in media from CD68sMSR transduced cells. It is possible that with a strong CMV promoter the cell's protein production capacity was exceeded and part of the protein remained unglycosylated (Resnick et al., 1993).

sMSR protein that had been produced in RAW 264.7 macrophages after adenovirus mediated gene transfer of CD68sMSR could inhibit degradation of ¹²⁵I-AcLDL and ¹²⁵I-OxLDL by 70% and 80%, respectively. sMSR produced in RAASMC after CMVsMSR transduction inhibited the degradation of ¹²⁵I-AcLDL by 70%-80% but the degradation of ¹²⁵I-OxLDL was inhibited only by 30-50%. Conditioned media from WHHL fibroblasts transduced with sMSR expressing AAV-was also tested in degradation assay and the *in vitro* degradation of ¹²⁵I-AcLDL was decreased by an average of 45% (35-84%).

A concentration of 700ng/ml-60µg/ml (ELISA) of sMSR per 12 h collection was produced in RAASMC after adenovirus mediated gene transduction of CMVsMSR compared to the 2µg/ml (dot blot) of sMSR produced after CD68sMSR transduction in RAW 264.7 cells. However, the degradation of AcLDL was not inhibited in proportion to the increased amount of sMSR. Dot blot is a rough method to analyze protein concentration and thus the concentration of sMSR after CD68sMSR transduction is an approximation and could undermine the concentration. However, it is also possible that there is a threshold limit after which an additional amount of sMSR does not result in an increased effect or possibly sMSR could not bind to some epitopes that are ligands to other modified LDL receptors. Alternatively, unglycosylated sMSR among glycosylated sMSR may have affected ligand binding.

Preincubation of sMSR with RAW 264.7 did not alter the degradation of ¹²⁵I-AcLDL indicating that sMSR could not form higher form oligomers with the native MSR or bind to any other molecule on the cell surface or that binding of sMSR to macrophages did not have an effect in terms of the degradation assay. Alternatively, sMSR binding to macrophages was too weak to endure incubation before the assay and sMSR was detached or degraded from the macrophage membrane. However, it was shown in adhesion test with THP-1 monocyte/macrophages and sMSR that sMSR could bind to a yet unidentified cell surface molecule during preincubation and subsequently inhibit monocyte binding to EA.hy 926 endothelial cells *in vitro* (Figure 6B and III). The difference in the behavior of sMSR in degradation assay and adhesion test may not be that surprising since different domains in the MSR are involved in ligand binding and adhesion. Ligand binding is mediated by the collagen like domain whereas adhesion as well as apoptotic cell binding, acid dependent ligand dissociation and trimerization are mediated by the α -helical coiled coil domain.

In the foam cell formation assay sMSR could inhibit lipid accumulation in RAW 264.7 cells which suggests that ligand binding to sMSR in the growth medium is the mechanism of the inhibition of the native scavenger receptor activity. This conclusion was supported by the fact that sMSR was also effective in reducing the degradation of AcLDL in MSR AI/II knock-out mouse macrophages via other putative scavenger receptors which presumably rely on the recognition of similar binding domains as that of MSR. In addition, since sMSR affects monocyte/macrophage adhesion it could be useful to test sMSR in other macrophage-accumulation diseases such as arthritis, especially since it has recently been shown that OxLDL and LOX-1 are involved in the pathogenesis of arthritis (Nakagawa et al., 2002).

sMSR *in vivo* (I- III)

When ^{125}I -AcLDL-sMSR complexes were injected to the mouse tail vein, most of the radioactivity was detected in the liver thirty minutes later. Since liver is the main organ involved in cholesterol secretion to bile, accumulation of modified LDL-sMSR complexes could target modified LDL out of the body as happens to HDL if SR-BI is overexpressed in the liver (Kozarsky et al., 1997). However, since the accumulation of ^{125}I -AcLDL-sMSR complexes was not followed longer than thirty minutes and because bile acid secretion and composition was not studied there is no certainty about this. It is equally possible, that complexes are phagocytosed / endocytosed by the liver cells where sMSR and modified LDL are detached and LDL can be secreted back to the circulation.

When CD68sMSR adenoviruses were studied *in vivo* in LDLR knock-out mice it was found that they could inhibit lesion formation but the effect was marginal – only 7-12% inhibition in *en face* aortic atherosclerosis after four months of Western type diet. With tissue specific promoters the virus is distributed the same way as with universal promoters but only (a) specific cell type(s) expresse(s) the transgene. Evidently, systemic gene transfer via tail vein with a tissue specific promoter was not optimal in studying the potential therapeutic effect of the sMSR. Therefore sMSR was subcloned under the CMV promoter and adenoviruses were produced.

When adenoviruses expressing sMSR under CMV promoter were injected in the mouse tail vein, gene expression was mainly detected in the liver although other tissues expressed sMSR as well (Table 5). This is in agreement with previous results (Hiltunen et al., 2000). Gene expression in the liver lasted at least four weeks which was the longest time point analyzed by RT-PCR and ELISA. Although a decline in the serum sMSR level by ELISA was seen five days after gene transfer we were able to detect sMSR in the serum throughout the experiment with up to 240 $\mu\text{g}/\text{ml}$ of sMSR three to five days after gene transfer compared to 16 $\mu\text{g}/\text{ml}$ of sMSR four weeks after the gene transfer. Transduction with first generation adenoviruses leads to a gene expression that can be detected from the liver up to three months after the gene transfer and the expression is dependent on the transgene and mouse strain used (Wang et al., 1997). According to Wang and coworkers the longest expression time is achieved with C57BL/6 mouse strain which is also the background mouse strain for our LDLR knock-out mice. RT-PCR detected sMSR mRNA in one third of the aortas examined but the effect of the sMSR on atherogenesis was found independently of the aortic expression of the transgene.

Since atherosclerosis progresses during decades we wanted to study a long term effect of sMSR in mice. Therefore, sMSR was subcloned into an AAV vector. AAV mediated gene transfer of sMSR caused a transient elevation in AST and AFOS that returned to baseline within eight and twelve weeks, respectively. Gene transfer did not affect CRP values. *In vivo* six months after intravenous gene transfer sMSR was mainly detected in the liver and spleen although the transgene was detected in other organs, including testis, as well. Earlier studies by others have shown that DNA can be detected in testis for less than 24h after intramuscular application of AAV. Therefore special caution may be required after i.v. administration of rAAV.

EGFP expression in the liver and detection of sMSR in mouse plasma showed that gene transfer lead to the expression of the transgene. sMSR was detected in the serum by ELISA two weeks after the gene transfer and the level of expression peaked two to three months after gene transfer. Trace amounts of sMSR were detected six months after the gene transfer. However, the standard deviation of sMSR in the serum was high. For example one month after the gene transfer one mouse had 0.95ng/ml and another mouse had 92.4ng/ml of sMSR in the serum. Differences in the sMSR expression level may be due to the initial low gene

transfer efficiency of some mice or could result from promoter silencing. In skeletal muscle the CMV promoter stays active for more than a year but in the liver the promoter is inactivated, possibly due to DNA hypermethylation (Prosch et al., 1996) and / or lack of endogenous activating transcription factors (Loser et al., 1998). Promoters also vary in efficiency and after intraportal administration of AAV there are 50-500 fold differences in expression level between different promoters. In any case, differences in the expression level of this magnitude indicate that a range of promoters should be tested for each experimental setting when AAV are used as gene transfer vectors. The WPRE in our AAV construct has been shown to increase transgene expression in mouse brain when associated with the PDGF-promoter (Paterna et al., 2000). The WPRE also increased expression levels with CMV promoter, but the result did not reach statistical significance. After tail vein injection of AAV most of the virus is detected in the liver. It is not clear whether using the WPRE together with the CMV promoter has improved gene expression in the liver. In addition, the route of virus administration affects transduction efficiency. Infusion of AAV into the portal circulation or direct injection to the liver is up to 100 times more efficient than gene transfer via tail vein – an observation also made in our laboratory. When 1×10^{11} AAVsMSR virusparticles were infused into the portal vein of WHHL rabbits up to 630ng/ml of sMSR protein was detected in the rabbit serum six months after the gene transfer (Vähäkangas *et al*, unpublished result). This is far more than what was detected in LDLR knock-out mice, even if the larger amount of virus in WHHL rabbits is taken into account. Also, the amount of virus used in our mouse study was relatively low compared to the experiments performed in other laboratories. Increasing the particle amount could have resulted in increased transduction efficiency (Nakai et al., 1998). It would be interesting to evaluate optimal virus titers and routes of administration (i.v., i.p., s.c., i.m., intra portal) for all virus vectors used. However, portal infusion with mice is difficult and direct injection to liver requires very high virus titers. We were able to obtain significant results and demonstrate the proof of principle with much less tedious method for mice.

CMVsMSR adenoviruses inhibited atherosclerotic lesion formation in LDLR knock-out mice. Four weeks after the gene transfer sMSR was able to reduce *en face* lesion area by 14% and six weeks after the gene transfer by 19% compared to LacZ transfected mice (Table 6, Figure 9). For a comparison, similarly analyzed LDLR / MSR A I/II double knock-out mice had a 45% reduction in the lesion area compared to LDLR single knock-out mice in Western type diet (data not shown) indicating that sMSR gene transfer inhibited about 40% of the MSR activity. This can be considered an excellent result in gene therapy experiment and most likely relies on the use of secreted molecule that augments the effect. AAV mediated gene transfer of sMSR caused a statistically significant 21% inhibition on aortic atherosclerosis at the end of the six month follow-up time. Although there was a tendency towards smaller lesion volumes with both viruses used we could not establish a statistically significant effect on lesion volume in the proximal aorta with either of the viruses. Immunostainings revealed that lesions on Western type diet were mainly composed of macrophages with LDL oxidation specific epitopes.

RAGE and MSR A I/II both bind AGE. Soluble RAGE inhibits accelerated atherosclerosis and stabilizes established lesions in diabetic ApoE knock-out mice. When we analyzed the complexity of the lesions by quantifying necrosis and cholesterol crystals in the aortic sections from LDLR knock-out mice transduced with sMSR expressing AAV and adenovirus or control viruses, sMSR did not stabilize lesion as compared to controls (unpublished results). The results obtained with RAGE were done in ApoE knock-out mouse model that had been subjected to streptozotocin that confers mice diabetic and increases the amount of

glycated proteins. In addition, ApoE knock-out mice have a dramatically increased cholesterol level of 494 mg/dl (12.8mmol/l) on chow diet and the level is further increased to 1821mg/dl (47.3mmol/l) on Western type diet. Even in the absence of complicating diabetes ApoE knock-out mouse model is a very aggressive model of atherosclerosis. Mice develop spontaneous atherosclerosis at the age of 10 weeks on chow diet (Palinski et al., 1994). LDLR knock-out mice on the other hand do not develop lesions on chow diet at all. In stead, they require Western type diet for at least 10 weeks to develop lesions that are comparable between individual mice.

When ApoE knock-out mice are cross bred with MSR A knock-out mice the resultant double knock-out mice have higher serum cholesterol concentration but less atherosclerosis. MSR A deletion in the LDLR knock-out mouse background results in fewer lesions (the effect is one third of that with ApoE knock-out mice) but MSR A deletion in the ApoE Leiden background results in more complex lesions. Therefore the deletion of the same gene in different mouse backgrounds may have different outcomes. It is possible that sMSR gene transfer in a more aggressive mouse model such as the ApoE knock-out model could have resulted in more profound effect on atherosclerosis and could have affected lesion composition and lesion volume in the proximal aorta as well.

However, most of the lipoprotein in ApoE knock-out mice is in VLDL fraction and it could be argued whether ApoE knock-out model is a relevant model for human atherosclerosis. We chose LDLR knock-out mouse model because its lipoprotein profile resembles human lipoprotein profile, with most of the lipoprotein being LDL and IDL. However, currently there are even better mouse models. Mouse liver is capable of secreting ApoB48 whereas human liver secretes solely ApoB100. Apobec-1 enzyme is responsible for the ApoB mRNA editing. ApoB only, Apobec1 and ApoBTg models in LDLR knock-out background are currently available mouse models that are the best for this kind of experiments. It could be feasible to repeat the results with either of these models (table 3).

C57BL/6 mice have single amino acid mutations in the MSR A I gene: one in the cytoplasmic domain, one in the spacer domain and four in the α -helical coiled-coil domain. One of the mutations in the α -helical coiled-coil domain prevent the antibody 2F8 binding to the MSR which usually inhibits AcLDL-stimulated cholesterol esterification in macrophages possibly by steric hindrance of ligand binding area in the collagen like domain. The authors did not study the effect of the mutations in the macrophage adhesion in B57BL/6 mice. However, in our experiment macrophages were abundant in lesions.

There has been debate about the role of oxidation specific antibodies in atherosclerosis. Some studies have shown an association between antibody titers and atherosclerosis (Salonen et al., 1992; Lehtimäki et al., 1999; Bellomo et al., 1995; Bergmark et al., 1995; Branch et al., 1994; Vaarala et al., 1995) while other studies indicate that immunization with OxLDL, MDA-LDL or LDL results in inhibition in lesion formation in mice and rabbits (Freigang et al., 1998). Although the mechanism of inhibition is not clear, it has been suggested that antibodies could form complexes with OxLDL and these complexes would be more readily cleared from the circulation. This would prevent OxLDL from penetrating lesions and/or OxLDL-OxLDL antibody complexes could be cleared by the macrophage Fc-receptors also in lesions. Also, the antiatherogenic effect of antibodies could result from T cell mediated mechanism (Freigang et al., 1998). It has also been shown that OxLDL antibodies can inhibit OxLDL degradation. It could be postulated that serum sMSR could result in enhanced removal of sMSR-OxLDL complexes from the circulation, prevent OxLDL penetration to lesions and affect OxLDL in lesions. sMSR could also inhibit OxLDL degradation.

In fact, our *in vitro* foam cell formation assay and degradation assay with MSR knock-out cells indicated that sMSR competed with transmembrane receptor for ligand binding but since mRNA was detected only in a few aortas it is not clear whether competition plays a role in the vessel wall. sMSR was also able to affect turnover of modified LDL *in vivo* indicating that sMSR was able to effect the tissue distribution of modified LDL (Figure 8). The failure to affect lesion volume in the proximal aorta (a site of early and aggressive atherosclerotic lesion formation) indicates that sMSR could not strip lipids from existing atherosclerotic lesions. Instead, it appears that sMSR is effective in inhibiting new lesion development. There was a statistically significant increase in the lesion area between LacZ transduced mice on Western type diet for 2.5 months and 4 months. There was not a significant change between 2.5 month LacZ group and 4 months sMSR groups indicating that the progression of atherosclerosis was inhibited not only between case and control but also between different time points. Furthermore, adenovirus mediated gene transfer resulted in the higher amount of sMSR in the mouse circulation compared to the AAV but AAV mediated gene transfer resulted in more profound effect on atherosclerosis possibly because gene transfer was done before the development of first lesions and because AAV mediated gene transfer resulted in long term gene expression. However, our studies did not establish the mechanism of atheroprotection *in vivo*. The next step is to evaluate the mechanism *in vivo*: is adhesion of monocytes to vessel wall inhibited, is cholesterol secretion to bile and out of the body increased, is there a change in LDL lipoprotein profile, and is there abnormal accumulation of lipids in other tissues in the long run.

PAF-AH (IV)

PAF is an inflammatory mediator that causes aggregation of platelets, polymorphonuclear leukocytes (PMN) and macrophages, stimulates monocyte secretion of cytokines and smooth muscle cell growth, activates platelets, neutrophils, monocytes, macrophages and vascular smooth muscle cells and is chemotactic for PMN leucocytes (Imaizumi et al., 1995; Prescott et al., 2000).

PAF-AH is an enzyme capable of hydrolyzing PAF and PAF-like lipids. It protects LDL from oxidation and inhibits oxidized lipoprotein and macrophage accumulation in lesions and inhibits neointimal formation in ApoE knock-out mice (Quarck et al., 2001). However, the reaction product is lysoPAF / lyso-PC which have been shown to be atherogenic. Lyso-PC is an essential component of OxLDL. Antibody titers against lyso-PC and OxLDL closely parallel in healthy individuals and incubation of lyso-PC with OxLDL antibodies almost completely abolish the activity of the antibody indicating that much of the reactivity of OxLDL is against lyso-PC (Wu et al., 1998). Lyso-PC in OxLDL is also a ligand to scavenger receptors. However, a recent article (Marathe et al., 2001) showed that lyso-PAF and lyso-PC are not inflammatory themselves but the inflammatory reaction results from a contaminating PAF or PAF-like lipids, supporting the evidence for beneficial role of PAF-AH in atherosclerosis and promoting to re-evaluate of some of the results gained earlier with lyso-PC.

Our results support the beneficial effect of PAF-AH on LDL oxidation and atherosclerosis related functions. Adenovirus mediated intravascular gene transfer of PAF-AH led to the production of functional PAF-AH protein that could be detected in the liver by immunohistochemistry and in the plasma and LDL by PAF-AH activity assay. PAF-AH was associated to LDL, protected LDL from oxidation during incubation with Cu^{++} which could be detected as decreased electrophoretic mobility in agarose gel. Increased PAF-AH activity in

LDL subjected to oxidation inhibited the degradation of LDL by murine macrophages and, more importantly, reduced foam cell formation in macrophages. Our results indicate that relatively small change in the amount of PAF-AH is capable of mediating effect on lipoprotein metabolism.

Carpenter *et al* (Carpenter et al., 2001) showed that PAF-AH inhibitors prevent OxLDL mediated cytotoxicity although the protective role was only evident after prolonged incubation. On the other hand, it has been shown that as low a concentration as $2-4 \times 10^{-2}$ U/ml of PAF-AH is able to eliminate the MM-LDL induced expression of MCP-1 and monocyte binding to endothelial cells (Watson et al., 1995). In addition, Watson and co-workers showed that in the presence of HDL the forming lyso-PC was transferred from MM-LDL to HDL. The authors proposed that the role of HDL might be to redistribute PAF-AH to LDL. However, it is also possible that if HDL accepted the lyso-PC also *in vivo* the effects of lyso-PC in LDL/OxLDL were less profound and the role of PAF-AH could be primarily protective. Therefore the timing and the site of the PAF-AH activity may determine the outcome. In the early phases of atherosclerosis (LDL oxidation, foam cell formation and fatty streak formation) PAF-AH may exert a protective role whereas in later stages and in more advanced plaques PAF-AH may promote cytotoxicity and apoptosis and subsequent release of macrophage enzymes and lipid constituents, creating lipid cores and possibly weakening the plaque. It is also worth emphasizing that great amounts of lyso-PC are also produced during LDL oxidation by oxidative mechanisms and during oxidative stress and lyso-PC produced by these mechanisms may constitute the bulk of lyso-PC in atherosclerosis.

Gene therapy and atherosclerosis

Current vectors are limiting factor in gene therapy. Low transduction efficiency, lack of regulation, immunogenicity, low cloning capacity, contamination with wild type viruses, technical problems in virus production and transduction as well as risk of mutations limit safety and effectiveness of gene therapy. Vector development is needed before gene therapy can be a realistic choice in clinical medicine. However, current gene transfer experiments are justified and needed to gain more information about the pathology of atherosclerosis and to evaluate different genes that could be used in clinical medicine in the future. Gene therapy could be efficient in treating monogenic diseases where the treatment strategy is obvious: gene therapy is used to transfer gene(s) in order to restore normal function of the defective gene. Alternatively gene therapy could be efficient in solving localized problems with easy access such as restenosis (Hedman et al., 2003) or solid tumours (Sandmair et al., 2000).

We have shown for the first time that atherosclerosis can be inhibited with soluble lipoprotein receptor sMSR during atherogenesis. Our results once again underline the importance of modified lipoprotein receptors (*I-III*) and the role of oxidation (*IV*) in atherogenesis. Despite the good results, it can be questioned whether the total 20% inhibition in atherosclerosis is relevant in terms of future clinical applications. Atherosclerosis is a diffuse disease involving vasculature around the body and it most often has polygenic background. There are several affected tissues with several genes and/or gene families participating in the pathogenesis and the pathology of the disease in molecular level is not fully characterized. It is not easy to determine what, how and when to treat.

Atherosclerosis evolves during decades, which means that short-term gene expression may not be sufficient but long term gene expression and integrating vectors are needed. This raises concern about the safety of the vectors. Also, prophylactic treatment of patients that could or

could not get atherosclerosis is ethically questionable since there are very effective drugs such as statins that improve the patients' prognoses.

However, the results obtained by others with sRAGE (Park et al., 1998) give reason to speculate whether sMSR also could have a role in stabilizing plaques and reducing atherosclerosis especially in diabetic patients. It would be interesting to study if sMSR gene transfer or sMSR protein administration (or another protein capable of affecting lipoprotein receptor function) could stabilize vulnerable plaques once detected for example by intravascular ultrasound (IVUS).

Furthermore, there is a population of patients who have a strong genetic risk of atherosclerosis such as FH patients. Heterozygous patients often respond to statin therapy. Homozygous patients with partially functioning LDLR can respond to statin therapy or to a combination therapy with statins, fibrates and niacin. Ezetimibe, an inhibitor of cholesterol absorption, in combination with atorvastatin or simvastatin has lowered the LDL cholesterol level in homozygous FH patients. However, there are still a number of patients who fail to reach satisfactory cholesterol level and have to rely on plasmapheresis or liver transplantation. Although pilot studies with *ex vivo* gene transfer of LDLR in FH has resulted in rather disappointing outcome, gene therapy remains as an attractive alternative treatment of the future. With improved vectors, combination therapy with LDLR and other antiatherogenic genes such as sMSR / PAF-AH could improve the prognosis of these patients in the future.

7 SUMMARY AND CONCLUSIONS

1. We have cloned a functional soluble macrophage scavenger receptor that inhibits the degradation of AcLDL and OxLDL by 70% and 80%, respectively. The degradation of AcLDL was inhibited also in the MSR type A I/II knock-out mouse macrophages indicating that sMSR may be able to inhibit lipid uptake also via other modified LDL receptors. In addition, sMSR was able to inhibit foam cell formation in murine macrophages (*I*).
2. Intravenous administration of sMSR encoding adenoviruses lead to transgene expression detected by RT-PCR for four weeks after the transduction. sMSR protein was detected in the liver by immunohistochemistry and in the plasma of LDLR knock-out mice by ELISA. sMSR transduction led to 14% reduction in the aortic atherosclerosis after 10 weeks and to 19% reduction in atherosclerosis after 16 weeks on atherogenic Western type diet (*II*).
3. Intravenous administration of sMSR encoding AAV led to the gene expression detected by a sensitive ELISA in plasma six months after the virus infusion. sMSR inhibited aortic atherosclerosis in LDLR knock-out mice under Western type diet by 21% without adverse effects during the six month follow-up. In addition, sMSR inhibited monocyte/macrophage adhesion to endothelial cells *in vitro* (*III*).
4. Intravascular infusion of PAF-AH adenoviruses led to the production of functional PAF-AH protein that 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 reduced migration in lipoprotein electrophoresis and a dose-dependent inhibition in degradation of OxLDL by murine macrophages. In addition, OxLDL from PAF-AH transduced animals was less likely to promote foam cell formation (*IV*).
5. In the future, it would be feasible to test the effect of sMSR in stabilizing vulnerable plaques in an animal model of accelerated atherosclerosis. In addition, the function of both sMSR and PAF-AH in combination with LDLR should be tested in the treatment of atherosclerosis in an animal model of familial hypercholesterolemia.

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