

Adeno-associated Virus-Mediated Gene Transfer of a Secreted Decoy Human Macrophage Scavenger Receptor Reduces Atherosclerotic Lesion Formation in LDL Receptor Knockout Mice

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Macrophage scavenger receptors (MSR) promote atherosclerotic lesion formation, and modulation of MSR activity has been shown to influence atherosclerosis. Soluble receptors are effective in inhibiting receptor-mediated functions in various diseases. We have generated a secreted macrophage scavenger receptor (sMSR) that consists of the bovine growth hormone signal sequence and the human MSR A I extracellular domains. sMSR reduces degradation of atherogenic modified low-density lipoproteins and monocyte/macrophage adhesion on endothelial cells *in vitro*. To test long-term effects of sMSR, atherosclerosis-susceptible LDLR knockout mice were transduced via the tail vein with an adeno-associated virus (AAV) expressing sMSR or control enhanced green fluorescent protein (EGFP), and a Western-type diet was started. Gene transfer caused a temporary elevation in alkaline phosphatase and aspartate amino transferase values without a change in C-reactive protein. sMSR protein was detected in the plasma of the transduced mice by a specific ELISA 6 months after the gene transfer. AAV-mediated sMSR gene transfer reduced atherosclerotic lesion area in the aorta by 21% ($P < 0.05$) compared to EGFP-transduced control mice. Even though eradication of established disease was not possible, atherosclerotic lesion formation could be modified using AAV-mediated gene transfer of the decoy sMSR.

Key Words: adenoviruses, gene transfer, scavenger receptor, LDL receptor knockout mice, macrophages

INTRODUCTION

Adeno-associated viruses (AAV) are small, nonenveloped parvoviruses [1] that have not been associated with human diseases and are considered nonpathogenic. AAV are able to infect both dividing and nondividing cells. AAV mediate stable gene expression and are suitable for diseases for which long-term transgene expression is needed. Wild-type AAV integrates in a site-specific manner into chromosome 19, but with recombinant AAV the site-specific integration is lost and viruses integrate randomly or remain episomal [2,3]. AAV can transduce a wide variety of cell types and transgene expression has been reported at least in the retina, skeletal muscle, vascular smooth muscle, and central nervous system.

Atherosclerosis is a disease that develops over a long period of time and the first manifestations of the disease often appear in middle age. Macrophage scavenger receptors (MSR) types A I and A II are members of the scavenger receptor family that consists of integral membrane glycoproteins capable of mediating uptake of modified lipoproteins [4]. MSR A I and A II are known to play important roles in the development of atherosclerosis [5,6]. They can mediate accumulation of modified low-density lipoproteins (LDL) to macrophages and cause foam cell formation [4,7]. In addition, they function as adhesion molecules [8] and receptors for apoptotic cells [9] and are involved in host immunity [7]. Modulation of MSR activity reduces atherosclerosis in knockout mice [10–12].

Soluble decoy receptors offer a means to antagonize receptor-mediated functions. We have earlier reported the construction of a soluble decoy protein that consists of the bovine growth hormone signal sequence and the human MSR A I extracellular domains [13]. After an adenovirus-mediated gene transfer secreted (s) MSR inhibits degradation of acetylated (Ac) LDL and oxidized (Ox) LDL and inhibits foam cell formation *in vitro* [13].

In this study we evaluated the safety, feasibility, and effectiveness of the AAV-mediated sMSR gene transfer in mice during a 6-month follow-up time. It was found that tail vein injection of the AAVsMSR led to a transgene expression that could be detected by enzyme-linked immunosorbent assay (ELISA) 6 months after the gene transfer and that AAV-mediated gene transfer of the sMSR was able to reduce atherosclerotic lesion area by 21% in hypercholesterolemic LDLR knockout mice.

RESULTS

Degradation and Adhesion Tests *in Vitro*

We verified expression of the sMSR after AAVsMSR transduction in fibroblasts, rabbit abdominal aorta smooth muscle cells (RAASMC), and HeLa cells *in vitro* using immunocytochemistry (data not shown). We analyzed functionality of the sMSR protein in cell culture using a lipoprotein degradation assay: RAW 264.7 cells were incubated with conditioned medium from AAVsMSR-transduced rabbit fibroblasts or control cells along with 10 $\mu\text{g}/\text{ml}$ of ^{125}I -acLDL for 14 h. In the presence of sMSR, degradation of ^{125}I -acLDL was decreased by 45% (Fig. 1).

We studied the influence of sMSR on monocyte/macrophage adhesion on endothelial cells by labeling THP-1 cells with calcein-AM and assaying adherent THP-1 cells with a fluorometer. Incubation of the fluorescently labeled THP-1 cells with sMSR inhibited their adhesion on OxLDL-activated endothelial cells by 54% (Fig. 1).

Clinical Chemistry

We injected LDLR knockout mice in the tail vein with 7.5×10^9 particles of recombinant AAVsMSR or control AAVEGFP. We collected blood samples at 4-week intervals and sacrificed the mice 6 months after the gene transfer. Gene transfer caused an elevation in the plasma alkaline phosphatase (AFOS) and aspartate amino transferase (AST) values that peaked 2 weeks after the gene transfer and returned to the baseline 3 months after the gene transfer (Fig. 2). However, AAV did not cause any acute infections since C-reactive protein (CRP) remained at baseline (Fig. 2).

A Western type of diet caused an expected elevation in the total cholesterol: 6.0 ± 2.15 mmol/L at baseline and 25.6 ± 2.95 and 26.6 ± 2.72 mmol/L 6 months after the gene transfer in EGFP and sMSR groups, respectively. Triglyceride levels were 2.09 ± 0.20 mmol/L at baseline and 1.22 ± 0.28 and 0.98 ± 0.25 mmol/L 6 months later for

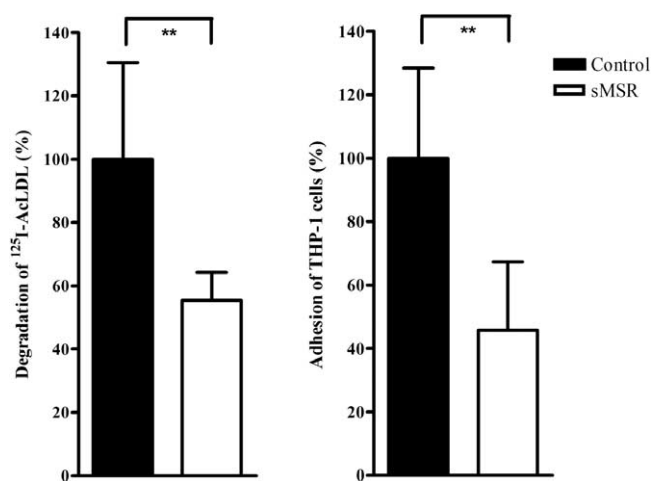


FIG. 1. Effect of sMSR on AcLDL degradation and monocyte/macrophage adhesion. Left: Degradation of ^{125}I -AcLDL. RAW 264 cells were incubated with ^{125}I -AcLDL (10 $\mu\text{g}/\text{ml}$) for 14 h with or without AAVsMSR-conditioned medium. Degradation is presented as a percentage of the ^{125}I -labeled lipoprotein degradation determined in the absence of any competitors. Right: Adhesion of monocyte/macrophage. Fluorescently labeled THP-1 cells were incubated with sMSR-conditioned medium and placed on EA.hy 926 cells for 1 h. Nonadherent cells were removed and adherent cells were measured with a fluorometer. All values are means \pm SD of nine determinations. $**P \leq 0.01$.

EGFP and sMSR, respectively. No significant differences were found in plasma total cholesterol and triglyceride levels between sMSR and EGFP groups during the study (Fig. 2).

Expression of sMSR *in Vivo*

We examined liver, spleen, lung, kidney, suprarenal gland, heart, striated muscle, testis, and epididymis with PCR. Liver, spleen, and suprarenal gland were frequently positive for the transgene. In addition, two testicles and one kidney were positive for the transgene (Fig. 3). We examined the sMSR expression in the aorta by RT-PCR. sMSR mRNA was found in the aortas of 3 mice 3 months after the gene transfer.

We analyzed the amount of sMSR produced *in vitro* after AAVsMSR-mediated gene transfer by ELISA. Conditioned medium collected from rabbit fibroblasts contained 180 ng/ml to 15 $\mu\text{g}/\text{ml}$ (average of 5 $\mu\text{g}/\text{ml}$) of sMSR protein per 24-h collection. Conditioned medium from RAASMC after AAVsMSR transfection contained 1–3.6 ng/ml (average of 2.5 ng/ml) of sMSR protein per 24-h collection. For a comparison, conditioned medium from RAASMC after adenovirus-mediated transfection contained 700 ng/ml to 60 $\mu\text{g}/\text{ml}$ (average of 25 $\mu\text{g}/\text{ml}$) of sMSR protein per 12-h collection (data not shown).

We also tested plasma samples from AAVsMSR-transfected mice by ELISA. sMSR expression could be detected in the plasma 2 weeks after the gene transfer (average 5.4 ng/ml) and reached a maximum level 3 months after the

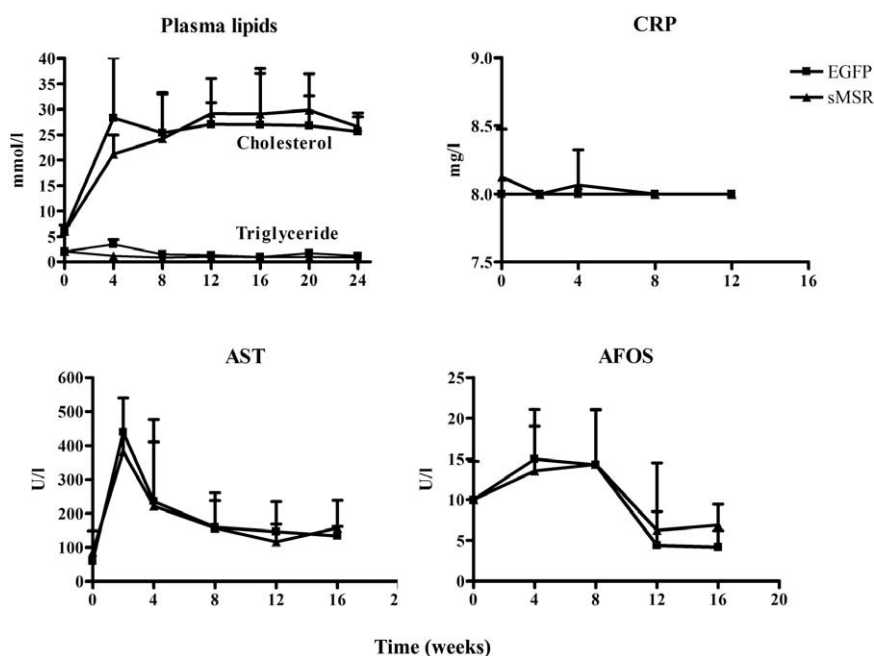


FIG. 2. Clinical chemistry. Mice were transduced via the tail vein with 7.5×10^9 particles of AAVsMSR or control AAVEGFP virus and plasma samples were collected. CRP, AFOS, AST, total cholesterol, and triglycerides were analyzed as indicated under Methods. No statistically significant differences were found between sMSR and control groups.

gene transfer (average 36.5 ng/ml) (Fig. 4A). Six months after the gene transfer we found up to 9 ng/ml (an average of 1.6 ng/ml) of sMSR protein in plasma. However, 5 of 12 examined mice did not express sMSR at the end of the experiment. sMSR level in control mice was below the detection limit of the ELISA.

We analyzed liver sections from transduced mice under fluorescence microscope with excitation at 488 and

360 nm for EGFP and DAPI, respectively. We detected a fluorescence signal from liver sections of the EGFP-transfected mice 6 months after the gene transfer (Fig. 4B).

Effect of sMSR Gene Transfer on Atherosclerotic Lesions

After sacrifice, we fixed the aortic arch and thoracic and abdominal aorta, pinned them out under the dissecting microscope, and evaluated the en face lesion area. In the control EGFP-transduced mice the atherosclerotic lesion area was $23.7 \pm 1.9\%$ ($n = 12$) and in the sMSR group $19.2 \pm 0.6\%$ ($n = 12$). Thus, sMSR reduced the lesion area in LDLR knockout mice aortas by 21% ($P < 0.05$) 6 months after the gene transfer (Fig. 5A). To study whether EGFP expression could affect atherosclerosis we examined aortas from LDLR knockout mice that had been on the Western-type diet for 6 months without any gene transfer. Atherosclerotic lesion area in the nontransduced mice was $23 \pm 6\%$ ($n = 6$) and did not differ from that in EGFP-transduced mice.

We measured atherosclerotic lesion volumes at the aortic valve level of the immunostained paraffin sections. However, there were no statistically significant differences in the lesion volumes (Fig. 5B). We also examined whether sMSR could have influenced the composition of the lesions by immunostaining for macrophages and oxidation-specific epitopes. We found no difference in the composition of lesions that were rich in macrophage-derived foam cells and oxidation-specific epitopes in both groups (Fig. 6).

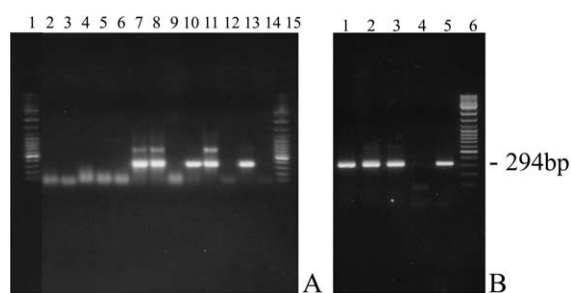
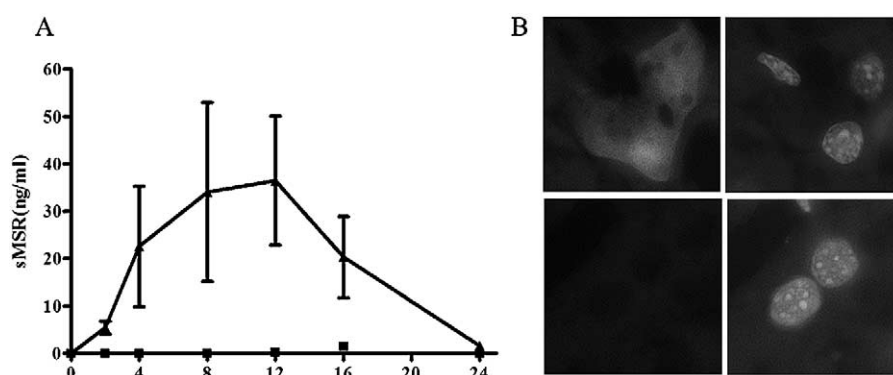


FIG. 3. PCR detection of sMSR transgene in LDLR knockout mice. Mice were transduced via the tail vein with 7.5×10^9 particles of AAVsMSR viruses and sacrificed 6 months later. DNA was isolated and analyzed with PCR. A PCR picture from one representative mouse is presented. (A) Lanes: 1, DNA ladder; 2, epididymis; 3, testis; 4, heart; 5, skeletal muscle; 6, lung; 7, suprarenal gland; 8, spleen; 9, kidney; 10, liver; 11, positive control from the first PCR; 12, negative control from the first PCR; 13, positive control from the nested PCR; 14, negative control from the nested PCR; 15, DNA ladder. (B) Total RNA was isolated from aortas of transduced mice and analyzed with RT-PCR. Lanes: 1–3, aortas from three different mice; 4, negative control from the nested PCR; 5, positive control from the nested PCR; 6, DNA ladder.

FIG. 4. sMSR expression *in vivo*. (A) Plasma sMSR concentration 6 months after the gene transfer by ELISA. Concentration is presented as ng/ml \pm SEM. Number of mice at each time point 3–12 in the sMSR group and 2–7 in the EGFP group. Detection limit of the assay is 0.5 ng/ml. (B) EGFP expression in mouse liver 6 months after the gene transfer as detected by fluorescence microscopy. Upper row, EGFP-, and lower row, sMSR-transfected mouse livers with EGFP (a and c) and DAPI (b and d). Original magnification $\times 40$.



DISCUSSION

Atherosclerosis is a complex process and the development of clinical disease often takes decades. MSR A I/II are involved in atherosclerosis. They mediate lipid accumulation during atherosclerosis [4,7,14–16] and serve as adhesion molecules [9]. Deletion of MSR A I/II inhibits atherosclerotic lesion formation by 23–28 and 60% in LDLR knockout mice and ApoE knockout mice, respectively [10,11]. However, depletion of the MSR A activity in ApoE3Leiden transgenic mice results in the development of more severe lesions as judged by their cellular composition [17], indicating that several factors influence atherosclerosis.

MSR mediates cation-independent adhesion in the presence of fetal calf serum and adhesion can be inhibited by MSR antibody 2F8. Adhesion is dependent on cellular activation state [8,18]. MSR also mediates macrophage adhesion to frozen spleen sections and to glycosylated collagen type IV [19,20]. Our finding that incubation of THP-1 cells with sMSR-containing medium inhibits THP-1 monocyte/macrophage adhesion on OxLDL-activated endothelial cells is in line with the earlier studies of MSR and could be an important factor in the *in vivo*

antiatherogenic effect of sMSR. In addition, we have shown earlier that sMSR inhibits foam cell formation *in vitro* [13]. We now report that AAV-mediated gene transfer of soluble decoy sMSR decreased the degradation of AcLDL by 45%. Therefore we have been able to deduce two important atherogenic functions of MSR A I/II, namely the lipid accumulation in macrophages and the monocyte/macrophage adhesion on endothelial cells. However, *in vivo* adhesion tests should be done before definite conclusions can be drawn about the effect on adhesion. Since mouse lesions are mostly derived from monocyte/macrophages it will be important to test the effectiveness of sMSR also in other animal models.

In vivo AAV-mediated gene transfer caused an elevation of the liver enzymes AFOS and AST. Liver enzyme values were normalized within 3 months of transduction. AAV did not result in acute infection as CRP remained at baseline. We also wanted to investigate whether an inflammatory reaction was induced in the liver by the gene transfer. We examined liver sections from sMSR- and EGFP-transduced mice and from nontransduced mice 2 and 3 months after the gene transfer by general histology and anti-macrophage immunostaining. No differences were detected between study groups (data not shown). Western-type diet was started at the time of the gene transfer, which elevated the cholesterol and triglyceride levels similarly in sMSR and EGFP groups. All together 70 mice were tail vein injected with AAV. Only 1 control mouse and 4 sMSR mice were lost within 24 h after transduction. The rest of the mice remained in good health until the end of the experiment.

After intravenous gene transfer sMSR was detected mainly in the liver and spleen but the transgene was observed in other organs as well. The gene expression lasts at least 6 months as analyzed by EGFP expression and ELISA, which showed an average of 1.6 ng/ml sMSR protein in the plasma of AAVsMSR-transduced mice. AAVsMSR gene transfer reduced *in face* aortic atherosclerosis in hyperlipidemic LDLR knockout mice by 21% during the 6-month follow-up time. We also examined aortic atherosclerosis in mice that had been on a Western-type

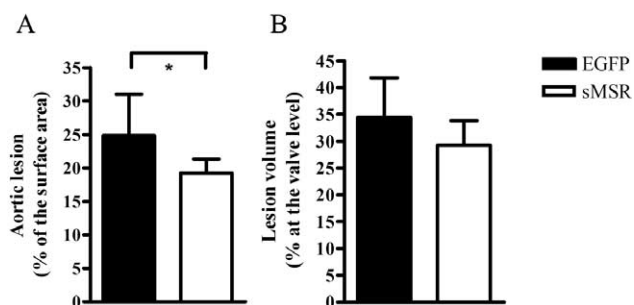


FIG. 5. AAVsMSR reduces atherosclerotic lesion area. Six months after the gene transfer mice were sacrificed and aortas were fixed, opened, and pinned out under the dissecting microscope. (A) Aortic lesion area was measured as a percentage of the whole aortic surface area with an image analyzer as described under Methods ($*P < 0.05$). (B) Lesion volume was analyzed at the aortic valve level, at which no significant difference was observed.

diet for 6 months without gene transfer. There was no difference in aortic atherosclerosis between EGFP-transduced mice and nontransduced mice, which indicates that AAVEGFP did not influence atherosclerosis but the reduction in atherogenesis was most likely due to sMSR.

sMSR did not have any statistically significant effects on the lesion volume in proximal aorta as analyzed from the histological sections at the aortic valve level. Immunostainings revealed that lesions were composed mainly of foamy macrophages. There were no differences in the presence of advanced lesions between sMSR and EGFP groups as analyzed from the histological sections (data not shown). The failure to affect lesion volumes in the proximal aorta indicates that atherogenesis at the valve level may depend on somewhat different processes than in the descending aorta. Accordingly, even in the total absence of MSR A in the LDLR knockout background atherosclerosis develops at the valve level [11]. Also, antiatherogenic properties of sMSR might have been better detected in a more aggressive mouse model such as ApoE knockout mice, whose atherogenesis depends more on macrophage functions and scavenger receptors than that in the LDLR knockout mice.

The 21% reduction in atherosclerosis gained here is modest compared to the MSR knockout mouse models, probably because the total elimination of the MSR activity by gene transfer methods is not possible. It can be questioned whether modifying only one of the lipoprotein receptor-mediated mechanisms by gene therapy is sufficient for the reduction of atherosclerosis and the results might be improved by combination therapy with other genes, such as inhibition of anti-inflammatory mediators or combining sMSR with gene transfer of other lipoprotein receptors. Familial hypercholesterolemia results from the mutation in the LDL receptor and causes severe atherosclerosis early in life. Liver-directed gene therapy that combines sMSR with LDL receptor could be more efficient than either one alone. Gene transfer of sMSR could also be used to transduce cells in blood vessels during transplantation procedures or in grafts during bypass operation.

Although AAV mediates a stable transgene expression, the choice of the promoter may be of great importance. The CMV promoter stays active in skeletal muscle for more than a year but in the liver the promoter is inactivated, possibly due to DNA hypermethylation [21] and lack of endogenous activating transcription factors [22]. When Xiao *et al.* compared the effectiveness of five different promoters after infusion of 10^{11} AAV particles into the portal circulation for liver-directed gene therapy, the highest levels of reporter gene α -1 anti-trypsin (α -1AT) expression were achieved using a retrovirus LTR promoter and the human albumin promoter. A CMV-enhanced β -actin promoter yielded intermediate levels of α -1AT but the lowest expression was detected with CMV and human phosphoglycerate kinase promoter [23]. The presence of the woodchuck hepatitis virus posttranscriptional regula-

tory element (WPRE) in our construct increases the transgene expression at least in mouse brain [24]. However, it is possible that selection of another type of promoter could have led to higher transgene expression.

Nevertheless, our results show that AAV-mediated gene transfer is safe and remains detectable for at least 6 months. The results also indicate that sMSR is able to affect atherosclerotic lesion formation and raises modest optimism about the possibility of gene therapy with soluble decoy sMSR.

METHODS

Production of recombinant adeno-associated viruses (rAAV). AAV vector psub-CMV-sMSR-flag-WPRE (AAVsMSR) encoding sMSR under the CMV promoter and WPRE was generated (Fig. 7) [24]. sMSR was digested from the pcDNA 3 vector with *HindIII* and *XbaI*, vector and insert were blunt-ended, and sMSR was subcloned into the psub-CMV-WPRE vector at the *MluI* site [13]. Adenovirus helper plasmid pBS-E2A-VA-E4, generated from pBluescript by inserting adenovirus 5 fragments VA1 and II, E2A, and E4 to provide helper functions, as well as the AAV packaging plasmid pAAV/Ad-rep(ACG), generated from pAAV/Ad28 by PCR-mediated mutagenesis of the Rep78 start codon from ATG to ACG, was used in the production of rAAV as described [24]. Briefly, 10 145-cm² plates of 293T cells grown at 80% confluency were cotransfected with AAVsMSR, pAAV/Ad-rep(ACG), and pBS-E2A-VA-E4 plasmids with the calcium phosphate method at the molar ratio 1:1:1. Fourteen hours later fresh DMEM containing 10% FCS was given. Cells were collected 48 h after transfection in their medium and rAAV were released from the cells by repeated freezing and thawing. rAAV were purified in iodixanol gradient (OptiPrep, Nycomed, Amersham Pharmacia Biotech, London, UK) as described [25]. Briefly, 15 ml of crude viral lysate was centrifuged against iodixanol gradient at 350,000g for 1 h at 18°C. Four milliliters of the virus band was aspirated and purified by heparan sulfate Sepharose HPLC. The titer of the virus was analyzed by slot blot. EGFP virus was generated as described [24].

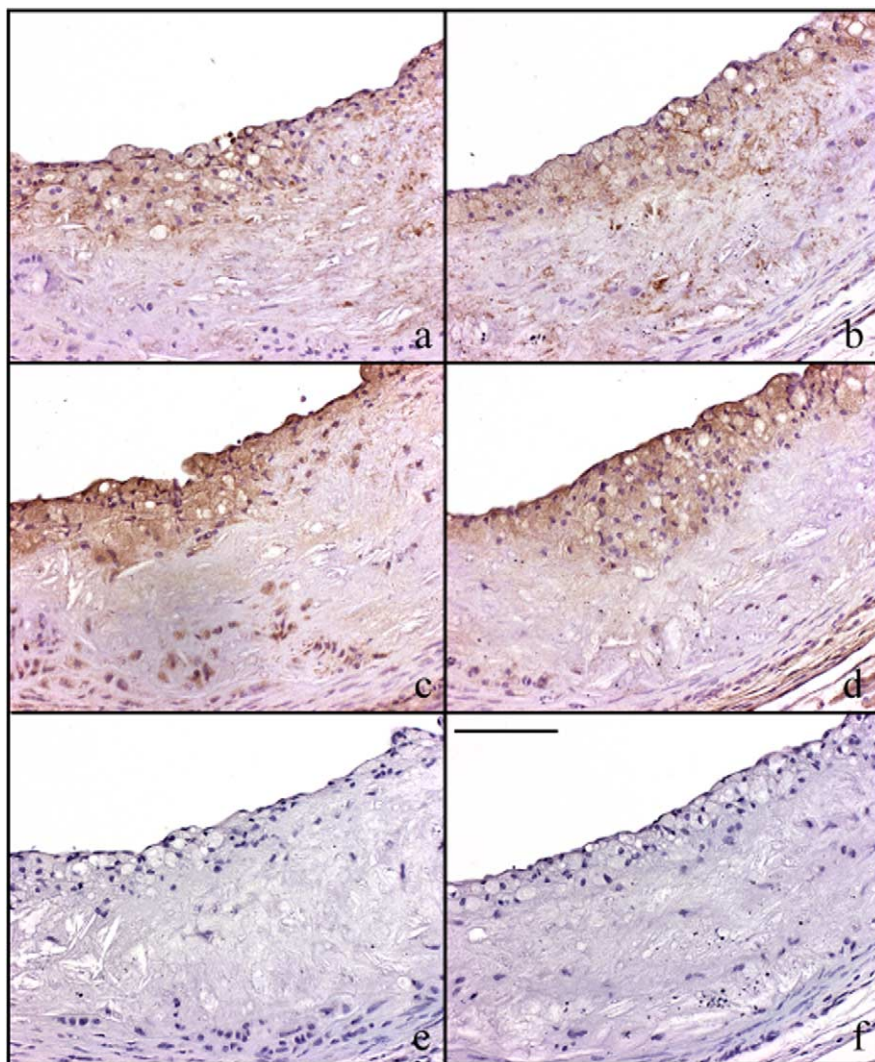
Cell culture. Watanabe heritable hyperlipidemic rabbit (WHHL) fibroblasts were grown in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin (5 U/ml, 5 μ g/ml, respectively). Cells (8×10^6) were plated on 10-cm plates. WHHL fibroblasts were transduced with AAVsMSR at 200 particles per cell and empty adenoviruses at m.o.i. 10 [26]. Negative control cells were transfected with empty adenovirus. All transfections were done in DMEM containing 2% FBS. Fresh medium containing 10% lipoprotein-deficient serum was changed 24 h after the transfection and medium was collected at 24-h intervals, filtered (0.45 μ m), and frozen in -20°C .

RAASMC were grown in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin (5 U/ml, 5 μ g/ml, respectively). Cells (8×10^6) were plated on 10-cm plates and transduced with recombinant adenoviruses expressing sMSR or control LacZ at m.o.i. 1000 [13]. Fresh medium containing 10% lipoprotein-deficient serum was given and medium was collected at 12-h intervals.

THP-1 cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM Hepes, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, 10% fetal calf serum, and penicillin/streptomycin (5 U/ml, 5 μ g/ml, respectively). Cells were split and/or medium was renewed twice a week. EA.hy 926 endothelial cells were grown in DMEM supplemented with 10% fetal calf serum, penicillin/streptomycin (5 U/ml, 5 μ g/ml, respectively), and HAT (100 μ M hypoxanthine, 0.4 μ M aminopterin, 16 μ M thymidine).

Isolation and modification of LDL. LDL was isolated from fasting plasma of healthy normolipidemic donors by sequential ultracentrifugation [27] and radioiodinated [28] before acetylation [29] or oxidation [27]. Specific activity of the labeled LDLs was about 250–800 cpm/ng protein.

FIG. 6. Representative lesion histology 6 months after the gene transfer. Aortic sections from AAVsMSR- (left column) and AAVEGFP- (right column) transduced mice were stained for macrophages (a, b, mMQ) and oxidation-specific epitopes (c, d, Mal-2); (e, f) nonimmune controls. Original magnification $\times 20$.



Degradation assay. Conditioned medium from AAVsMSR-transfected cells was used for lipoprotein degradation assay [27]. RAW 264.7 macrophages were incubated with $10 \mu\text{g/ml}$ ^{125}I -AcLDL and conditioned medium for 14 h. Trichloroacetic acid-soluble noniodide radioactivity in the medium was measured with a gamma counter [27].

Adhesion test. EA.hy 926 cells (150,000) were seeded on 24-well plates. Endothelial cells were activated for 24 h with $100 \mu\text{g/ml}$ OxLDL and THP-1 cells with 100 nM PMA. Activated THP-1 cells were labeled with $5 \mu\text{M}$ calcein-AM (Molecular Probes, Eugene, OR) for 1 h in RPMI 1640 medium without serum. Nonfluorescent calcein-AM is lipophilic and is cleaved by intracellular esterases to yield charged fluorescent calcein. The excitation and emission wavelengths for calcein molecule are 480 and 530 nm, respectively. Fluorescently labeled cells were washed three times with PBS and divided to different tubes. Labeled cells were incubated for 30 min with conditioned sMSR or LacZ medium collected from adenovirus-trans-

duced RAASMC. Cells were centrifuged and the pellet was resuspended in RPMI 1640 medium without phenol red. Cells (60,000–100,000) were plated on top of the activated EA.hy 926 cells and incubated for 1 h. They were washed three times with PBS and covered with RPMI 1640 without phenol red. Fluorescent activity of the adherent cells was measured with a fluorometer (Perkin-Elmer HTS 7000 Plus Bio Assay Reader, Palo Alto, CA).

Enzyme-linked immunosorbent assay. ELISA plates were coated overnight at $+4^\circ\text{C}$ with $100 \mu\text{l}$ of anti-flag M2 antibody (concentration $10 \mu\text{g/ml}$) (Sigma, St. Louis, MO), diluted in 0.1 M NaHCO_3 (pH 9.5), and washed with PBS–0.05% Tween 20 three times prior to being blocked with 1% bovine serum albumin (BSA)–PBS for 1 h at room temperature. Plates were washed three times with PBS–Tween 20. A standard curve ranging from 0.1 ng/ml to $10 \mu\text{g/ml}$ was prepared from column chromatography-purified cell culture medium according to the manufacturer's instructions. Standard samples, conditioned medium, or mouse plasma samples diluted in 1% BSA– 0.5 M NaCl –PBS–0.05% Tween 20 were incubated on the plates for 2 h at room temperature. Plates were washed and incubated with polyclonal goat anti-scavenger receptor antibody (Chemicon International, Temecula, CA) at the dilution of 1:2000 for 1 h. Plates were again washed and incubated with HRP-conjugated anti-goat antibody (Chemicon International) at the dilution of 1:4000. After being washed, the plates



FIG. 7. AAV vector used in the study.

were incubated with peroxidase substrate (3,3',5,5'-tetramethylbenzidine as a chromogen) at 100 μ l per well for 30 min in the dark. Color development was stopped with 0.5 M H₂SO₄ at 100 μ l per well. Absorbances were measured at 450 nm (Multiscan) and compared to the standard curve. The detection limit of the ELISA was 0.5 ng/ml.

Animal experiments. LDLR knockout mice [30] were used in the study. At the age of 2 months the mice were tail vein injected with 7.5×10^9 AAVsMSR particles, and a Western-type diet containing 21% fat (wt/vol), 0.15% cholesterol (wt/wt), and 19.5% casein (wt/wt) without sodium cholate was started (Harlan Teklad, Teklad Adjusted Calories Western-Type Diet, Madison, WI) [31]. Diet and water were provided *ad libitum*. Before the start of the Western-type diet LDLR knockout mice did not have atherosclerotic lesions in the aorta. Animals were anesthetized using fentanyl-fluanisone (3.15 and 10 mg/kg)/midazolam (5 mg/kg). Blood samples were collected at 4-week intervals and mice were sacrificed 6 months after the gene transfer. Samples for genomic PCR and RT-PCR were taken, frozen in liquid nitrogen, and stored at -70°C . Mice were perfused with PBS and 4% paraformaldehyde for 1 min, fixed for 1–2 h in the same fixative, and immersed in PBS overnight [32]. Tissues were embedded either in paraffin or in OCT (Tissue-Tek, Zoeterwoude, Netherlands) and processed for histology. All animal experiments were conducted in accordance with the guidelines of the Experimental Animal Committee of Kuopio University. Plasma AFOS and AST were analyzed with Ecoline 25 (Merck, Rahway, NJ) and read with an EPOS 5060-analyzer (Eppendorf, Hamburg, Germany). Total cholesterol and triglycerides were analyzed with the CHOD-PAP method (Merck) and the results were read with the EPOS 5060. CRP was analyzed with QuikRead (Orion Diagnostic, Espoo, Finland).

Analysis of atherosclerotic lesions. Fixed aortas were opened and pinned out under a dissection microscope [33]. En face atherosclerotic lesions in the aortic arch and in the thoracic and abdominal aorta were measured as a lesion percentage of the total aortic area with an image analyzer and the MCID-M4 program (Imaging Research, Inc., Ontario, Canada). Lesion area from each aorta was measured in a blinded fashion three times and average values are reported. After the analysis opened aortas were embedded in paraffin, 8- μ m sections were cut, and aortic sections were stained with hematoxylin and eosin or immunostained. Lesion volumes were analyzed from the aortic valve level without Oil-Red-O staining, taking into account the whole lesion area surrounded by the vascular ring using the Image-Pro Plus 3.0 program (Media Cybernetics, MD).

Immunostainings. Eight-micrometer paraffin sections were used for immunocytochemical detection of macrophages (mMQ; Accurate Chemical & Scientific Corp., Westbury, NY; dilution 1:5000) and oxidized epitopes (Mal-2 [34]; dilution 1:100) from aortic sections. Twelve-micrometer OCT sections were used for detection of EGFP from liver sections. All immunostainings were done as described [32].

PCR. RNA and genomic DNA and total RNA were isolated with TRIzol compound (Gibco, Carlsbad, CA) from collected tissues. RNA was incubated with DNase (Promega, Madison, WI), for 30 min at 37°C and the enzyme was inactivated with EGTA and heating at 65°C for 10 min, followed by phenol–chloroform extraction. cDNA was synthesized from the total RNA with Superscript II RNase H-free reverse transcriptase (Gibco) and random primers (Promega). Amplification of the transgene was performed from DNA samples, cDNA samples, and also 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 for 5 min) was followed by 40 cycles (95°C for 45 s, 57°C for 30 s, 72°C for 60 s) with a final extension of 10 min at 72°C . Two microliters of the first PCR product was subjected to nested PCR with primers 5'-GGACGACGATGACAAGGCGG-3' and 5'-TTGCATCCCAT-GTCCCTGG-3', both annealing to the MSR (95°C 3 min and 40 cycles of 95°C for 45 s, 72°C for 90 s, with final amplification for 10 min at 72°C).

Statistical analyses. Independent samples *t* test or Mann–Whitney test were used to evaluate statistical significance. $P < 0.05$ was considered

statistically significant. Numerical values for each measurement are shown as means \pm SD or \pm SEM.

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