Adenovirus-Mediated Gene Transfer of a Secreted Form of Human Macrophage Scavenger Receptor Inhibits Modified Low-Density Lipoprotein Degradation and Foam-Cell Formation in Macrophages

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Background—Macrophage scavenger receptors (MSRs) play an important role in the pathogenesis of atherosclerosis. Therefore, local modulation of MSR activity could have a beneficial effect on atherogenesis.

Methods and Results—We cloned a secreted “decoy” MSR (sMSR) that contains an extracellular portion of the human MSR type AI and constructed an adenoviral vector that directs high-level expression of sMSR in macrophages under the control of the human CD68 promoter. Expression of the sMSR protein inhibited the degradation of 125I-labeled acetylated LDL and oxidized LDL by murine macrophages up to 90%. sMSRs also reduced acetylated LDL degradation in MSR knockout mouse peritoneal macrophages by 60% to 80%, which suggests that the decoy construct can compete for the uptake mediated via other related scavenger receptors. In addition, sMSRs inhibited foam-cell formation in murine macrophages in the presence of cytochalasin D. The mechanism of inhibition is through ligand binding to the sMSRs, which prevents the ligand binding to MSRs on cell membranes.

Conclusions—The demonstration that recombinant adenovirus–mediated gene transfer of decoy sMSRs can block foam-cell formation suggests a possible new strategy for gene therapy of atherosclerosis and for the treatment of lipid accumulation after arterial manipulations. 

Key Words: viruses ■ genes ■ receptors ■ lipoproteins ■ cells

Macrophage scavenger receptors (MSRs) type AI/II are trimeric membrane glycoproteins that are involved in the deposition of lipids in the arterial wall during atherogenesis.1–5 They also mediate cation-independent adhesion of macrophages in vitro.6 MSRs belong to a family of receptor proteins that have cysteine-rich extracellular domains and exhibit a broad ligand-binding specificity.1–5 They can efficiently bind and internalize several polyanions and modified proteins, such as acetylated (ac) LDL and oxidized (ox) LDL, maleylated BSA, several polysaccharides, endotoxin, some phospholipids, and polynucleotides.1–5 Common to all MSR ligands is that they are negatively charged macromolecules.4,5 Members of the receptor family may also participate in host-defense functions.5–7

MSR type AI/II receptors have been implicated in the deposition of lipids in the arterial wall during atherogenesis.8–10 Because MSR is not downregulated by excess cholesterol accumulation in the cell, it can mediate continuous accumulation of lipids in the lesion macrophages.3–5 MSR expression is also strongly induced during lesion development.11 Studies with MSR AI/II knockout mice and their cells show that MSR accounts for 80% of the degradation of acLDL and 30% to 50% of the degradation of oxLDL,10 which is one of the MSR ligands in the vessel wall.12 Lesion formation is also significantly reduced in MSR AI/II knockout mice.10 Therefore, modulation of the MSR activity could have an important effect on atherogenesis.

We constructed a chimeric fusion protein that consists of the bovine growth hormone signal sequence and the human MSR AI extracellular domain. This secreted MSR (sMSR) was cloned into an adenoviral vector, and sMSR recombinant adenoviruses were produced under the control of the promoter of macrophage gene CD68. Adenoviruses were used to transduce murine macrophages, and the conditioned media were shown to reduce degradation of acLDL and oxLDL by 70% to 90% and to inhibit macrophage foam-cell formation.

Methods

Materials
pRc/CMV plasmid was from Invitrogen and pNASSm plasmid from Clontech. All cell culture reagents were from Gibco BRL. Oligo-dT cellulose, 5-bromo-4-chloro-3-indolyl-phosphate, and 4-nitro blue

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Southern and Northern Blots

Adenovirus genomic DNA was digested with HindIII and analyzed in a Southern blot with a random-primed DNA probe specific for both MSR and sMSR. RAW 264 macrophages and rabbit aortic smooth muscle cells (SMCs) were transduced with SSMR adenoviruses at 1000 MOI. Uninfected control plates were treated similarly to the transduced plates. mRNA was isolated with oligo-dT cellulose resin and analyzed in a Northern blot with a random-primed probe specific for sMSR. The membrane was stripped and reprobed with a human β-actin probe.

Western Blot

RAW 264, ECV 304, and NIH 3T3 cells and SMCs were transduced with sMSR adenoviruses; the medium was changed to Optitrem containing 0.5% FBS, and the cells were grown for 48 hours. Medium was collected and lyophilized, electrophoresed under reducing and nonreducing 10% SDS-PAGE, and transferred to a nitrocellulose membrane. sMSR was detected with a mouse anti-Flag monoclonal antibody (M5) according to the manufacturer’s instructions.

Poly(G) Resin Incubation Test

Control medium and medium containing sMSR were incubated with poly(G) resin overnight as described. Medium was electrophoresed under nonreducing 8% SDS-PAGE and transferred to a nitrocellulose membrane, and sMSR was detected as described above.

Dot-Blot Assay

The amount of sMSR protein in the transfection medium was measured with a dot-blots assay from several dilutions of the transfection medium, which were spotted onto a nitrocellulose membrane with standards ranging from 0.01 to 200 ng/100 μL. Proteins were detected with anti-Flag M5 antibody and quantified with an image analyzer and MCID-M4 program (Imaging Research Inc). Generation of the purified sMSR-AI protein standard will be described in detail elsewhere (Gough P.J. et al, manuscript in preparation).

Isolation and Modification of LDL

LDL was isolated from fasting plasma of healthy normolipidemic donors by sequential ultracentrifugation and radioiodinated before acetylation or oxidation. Specific activity of the labeled LDLs was 90 to 250 cpm/ng protein.

Degradation Assay

RAW 264 macrophages were transduced with sMSR adenoviruses, and Optitrem containing 10% lipoprotein-deficient serum (LPDS) was added. Conditioned medium was collected every 12 hours and used for lipoprotein degradation and competition assays. Untransduced control plates were treated similarly and used as controls. In earlier experiments, it was determined that conditioned medium from lacZ control adenovirus –transfected cells did not have any effect on the degradation of acLDL in macrophages. RAW 264 cells and peritoneal macrophages from MSR-A knockout mice and MSR-A/ LDL receptor double-knockout mice were incubated with 10 μg/mL of 125I-labeled acLDL or 125I-labeled copper oxLDL and conditioned medium for 9 hours. After incubation, the media were analyzed for LDL degradation products.

Foam-Cell Formation

RAW 264 cells were plated on chamber slides and incubated for 24 hours with Optitrem/10% LPDS containing 100 μg/mL acLDL and 4 μg/mL phagocytosis inhibitor cytochalasin D with or without sMSR (2.5 μg/mL). After the incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde, and stained with oil red O in 60% isopropanol, 0.4% dextrin and hematoxylin.

Cloning of the sMSR and Adenovirus Plasmids

sMSR was constructed in 3 steps (Figure 1). First, a 109-bp fragment was created by polymerase chain reaction (PCR) coding for 28 amino acids of the bovine growth hormone signal sequence with HindIII and XbaI sites and an Eco47III site encoding the last 2 amino acids of the signal peptide. This fragment was cloned into pRc/CMV plasmid. Second, oligonucleotides encoding a “Flag” epitope (DYKDDDDK), and an Flag epitope were synthesized. This insert and the vector from step 1 were ligated together to form an sMSR. CD68 promoter and sMSR were subcloned in an adenoviral plasmid and used for production of sMSR recombinant adenoviruses. Adenovirus genome is divided into 100 map units, each map unit corresponding to 360 bases. Numbers shown in plasmid and adenovirus constructs correspond to these map units (diagrams are not to scale; see text for details).

Generation of Adenoviruses

Adenovirus plasmid psMSRA-1 was linearized and cotransfected with sub360 adenovirus genomic DNA in 293 cells by CaPO4 precipitation. Viruses from 1 expanded plaque were used for a large-scale preparation of the recombinant adenoviruses. Purified virus preparation was analyzed for the absence of toxicity, wild-type viruses, microbiological contaminants, and lipopolysaccharide as described.

Figure 1. Construction of sMSR plasmid (psMSRA-1) and adenovirus. Bovine growth hormone signal sequence (BGSS), Flag epitope, and extracellular portion of human MSR cDNA were ligated together to form an sMSR. CD68 promoter and sMSR were subcloned in an adenoviral plasmid and used for production of sMSR recombinant adenoviruses. Adenovirus genome is divided into 100 map units, each map unit corresponding to 360 bases. Numbers shown in plasmid and adenovirus constructs correspond to these map units (diagrams are not to scale; see text for details).

tetrazolium chloride were from Boehringer-Mannheim. 125I and nylon membrane were from Amersham, nitrocellulose membrane from Bio-Rad, and [3H]ICTP from New England Nuclear. Poly(G) conjugated resin, cytochalasin D, and anti-Flag monoclonal antibody M5 were from Sigma. RAW 264 mouse macrophages (TIB-71), ECV304 cells (CRL-1998), and NIH 3T3 fibroblasts (CRL-6361) were obtained from ATCC. Chamber slides were from LabTek Brand, Nunc International.

Control medium and medium containing sMSR were incubated with poly(G) resin overnight as described. Medium was electrophoresed under nonreducing 8% SDS-PAGE and transferred to a nitrocellulose membrane, and sMSR was detected as described above.
Organ Distribution of sMSR–$^{125}$I-acLDL Complex

Medium without or with sMSR protein (2.5 μg) was combined with 25 μg of $^{125}$I-acLDL for 30 minutes and injected into the tail vein of anesthetized MSR-A/LDL receptor double-knockout mice (n=4) in a total volume of 200 μL (2.1×10$^7$ cpm). Mice were euthanized 30 minutes later, and organs were analyzed for radioactivity as described.

Results

To study the effects of “decoy” scavenger receptor on the metabolism of modified LDL in macrophages, we cloned a soluble sMSR that contains the extracellular domains of human MSR A1 and produced replication-deficient adenoviruses in which transgene expression is controlled by the promoter of the macrophage gene CD68 (Figure 1). Because adenoviruses undergo RNA splicing during their replication cycle, Southern analysis was performed to confirm the presence of the full-length transgene (Figure 2a). An expected 7.9-kb HindIII fragment containing the 4.6-kb expression cassette was visualized by hybridization with MSR probe, whereas wild-type adenovirus (lane 3) did not hybridize with MSR probe.

To confirm that the DNA was transcribed to mRNA RAW 264 cells and SMCs were transduced with sMSR adenoviruses, mRNA was isolated and analyzed on Northern blot. A 1.5-kb sMSR fragment was detected in the transduced RAW 264 cells and SMCs. Controls show that the probe is specific for the sMSR (Figure 3a). The same membrane was stripped and hybridized with β-actin probe to confirm that similar amounts of mRNA were loaded on each lane (Figure 3b).

Western blot analysis was performed with RAW 264 macrophages, SMCs, NIH 3T3 fibroblasts, and ECV 304 cells transduced with sMSR adenoviruses, and lyophilized medium was analyzed in SDS-PAGE (Figure 4). Easily detectable amounts of sMSR protein were found in RAW 264 and ECV 304 cell supernatants. Fibroblasts and SMCs expressed only a barely detectable amount of sMSR protein (Figure 4). Most of the sMSR protein was present as a monomer and a dimer, but some trimer was also detected. Quantitative analysis based on a dot-blot assay from the
conditioned medium indicated that RAW 264 macrophages and ECV 304 cells produced 0.1 to 2.5 ng/μL sMSR protein in the medium during a 12-hour incubation. Without concentration, the production of sMSR protein in SMCs and NIH 3T3 fibroblasts was below the reliable detection limit of the dot-blot assay (1 ng). Under reducing conditions, almost all the sMSR protein from RAW 264 cells was detected as a monomer (Figure 4).

We hypothesized that the sMSR binds to modified LDLs in the growth medium and thus prevents the ligand binding to native scavenger receptors. To test this, we incubated the sMSR medium with poly(G) conjugated resin. Original sMSR medium, poly(G) resin–incubated medium, and proteins released from the poly(G) resin were analyzed with a Western blot along with the similarly treated control medium. The poly(G) resin was able to bind almost all (>90%) of the sMSR from the medium (Figure 5).

Functional analysis of the sMSR protein was performed with a lipoprotein degradation assay (Figure 6). 125I-acLDL (10 μg/mL) was added to the conditioned medium and incubated with cells for 9 hours. In the presence of sMSR, the rate of degradation of 125I-acLDL decreased by 70% to 90% (Figure 6a). The decrease was comparable to a 15-fold excess of unlabeled acLDL or 30-fold excess of oxLDL (data not shown). A similar decrease in the degradation was also seen with 125I-labeled oxLDL: sMSR was able to decrease the degradation of oxLDL by 60% to 80% (Figure 6b). The decrease was comparable to a 30-fold excess of unlabeled oxLDL or acLDL (data not shown). sMSR was also able to reduce the degradation of 125I-acLDL in peritoneal macrophages isolated from either MSR-A knockout mice (Figure 6c) or MSR-A/LDL receptor double-knockout mice (data not shown) by 60% to 80%. On the basis of the quantitative dot-blot assay (see above), it was calculated that the conditioned medium used for the competition studies contained 2.5 μg/mL sMSR protein versus 10 μg/mL labeled acLDL. If molecular weights of 2.5 × 10^6 Da for acLDL and 135 kDa were assumed for dimeric sMSR, the conditioned medium contained ~5 sMSR molecules for every LDL particle.

We next tested the possibility that part of the reduction in the degradation of modified LDLs could have been due to the interaction of the sMSR with the native scavenger receptor on cell membranes. sMSR-containing medium was incubated with RAW 264 cells for 9 hours and then replaced with control medium containing 125I-acLDL. However, preincubation did not decrease 125I-acLDL degradation, whereas simultaneous addition of the sMSR and 125I-acLDL decreased the degradation by 70% (Figure 7).

In experiments with 125I-labeled LDLs, the sMSR was able to decrease the degradation of oxLDL and acLDL when the modified LDL was present in small quantities (ie, 10 μg/mL). To test whether the sMSR could inhibit the uptake of larger quantities of modified lipoproteins, we tested the effect of
sMSR on foam-cell formation in RAW 264 macrophages. Cells were incubated with 100 μg/mL of acLDL and 4 μg/mL of cytochalasin D for 24 hours in absence (A) or presence (B) of sMSR medium. There was a marked decrease in lipid accumulation in cells incubated with sMSR medium. Oil red O staining (magnification ×20).

Organ distribution of sMSR–125I-acLDL complex was evaluated in vivo by injecting the complex into the tail vein of MSR-A/LDL receptor double-knockout mice. The results suggest that the accumulation of the complex was enhanced in liver compared with mice injected with 125I-acLDL alone (Figure 9).

**Discussion**

We have engineered a recombinant adenovirus that expresses a secreted form of human MSR under the control of the human macrophage CD68 promoter. It was found that sMSR effectively inhibited lipid accumulation and foam-cell formation induced by modified LDL in RAW 264 macrophages.

Several transmembrane receptors for growth factors and cytokines have a soluble counterpart that may participate in the regulation of growth hormone or cytokine effects by preventing the ligand from interacting with the transmembrane receptor. Secreted proteins have also been used in therapeutic studies to block the activity of the native transmembrane receptor. Thus, secreted decoy receptors offer potential new tools to influence pathological processes in vivo. Major problems with this approach have been the low affinity of engineered soluble receptors for physiological ligands and inability to achieve high enough concentrations of soluble decoy receptors in target tissues. Also, it may be difficult to obtain long enough expression of the transgene in the target tissue and avoid potential immunological problems related to repeated adenoviral gene transfer. However, secreted receptors could still prove useful in diseases in which even a relatively small amount of protein delivered locally could have a therapeutic effect and in which the most important therapeutic target is to clear the ligand from the treated area.

Adenoviral gene transfer of the sMSR was chosen because it was recently shown that catheter-mediated delivery of adenoviral vectors can lead to 5% transfection efficiency in human atherosclerotic arteries, which may be enough to achieve clinical effects with secreted transgene products. Soluble sMSR cloned in this study was placed under the control of the CD68 promoter, which directs a high expression level in macrophages. This was considered desirable because most of the scavenger receptor activity in human atherosclerotic lesions is expressed by macrophages. Transcriptional targeting of adenoviral gene transfer to certain cell types is also an additional safety feature in the use of recombinant viruses. The 2.9-kb CD68 promoter does not have a complete macrophage locus control region, and it was found that the promoter was also active in ECV 304 cells, whereas only a weak expression was detected in fibroblasts and SMCs. Expression in endothelial-like cells was considered beneficial in terms of possible local in vivo applications in the treatment of atherosclerosis and restenosis. Thus, the engineered sMSR adenovirus was able to direct clearly detectable transcriptional targeting to cell types involved in the processing of modified LDL in the arterial wall.

Poly(G) resin was able to bind almost all of the sMSR in the medium, indicating that sMSR presents ligand binding characteristics similar to those of the native MSR. Similar
binding characteristics have previously been reported by Resnick et al.17 and by Andersson et al.27 However, there are no reports about the ability of sMSR to show biological effects on macrophages or to prevent foam-cell formation. The findings suggest that ligand binding to sMSR in the growth medium is the mechanism of the inhibition of the native scavenger receptor activity. This conclusion is further supported by the fact that sMSR was also effective in reducing the degradation of acLDL in MSR-A knockout mouse macrophages in vitro via other putative scavenger receptors that presumably rely on the recognition of binding domains similar to those of MSR.

Our results suggest that sMSR may be a useful tool for studies regarding atherogenesis and macrophage functions. Because MSRs play an important role in foam-cell formation in vivo,8–10 it is important to note that sMSR inhibited foam-cell formation in RAW 264 macrophages even in the presence of high concentrations of modified LDL. Our results also suggest that in vivo, the accumulation of sMSR-modified LDL complex is enhanced in liver. This should be beneficial, because the liver is the major organ responsible for the secretion of excess cholesterol from the body. It is also possible that sMSR may affect monocyte/macrophage adhesion in progressive atherosclerotic lesions. The results warrant in vivo evaluation of the adenosinemediated gene delivery of sMSR for the treatment of atherosclerosis and lipid accumulation after intravascular manipulations.

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