Lack of a direct role for macrosialin in oxidized LDL metabolism

Maria C. de Beer, Zhenze Zhao, Nancy R. Webb, Deneys R. van der Westhuyzen, and Willem J. S. de Villiers[1]

Department of Internal Medicine, University of Kentucky Medical Center, Lexington, KY 40536; and Department of Veterans Affairs Medical Center, Lexington, KY 40511

Abstract **Murine macrosialin (MS), a scavenger receptor family member, is a heavily glycosylated transmembrane protein expressed predominantly in macrophage late endosomes. MS is also found on the cell surface where it is suggested, on the basis of ligand blotting, to bind oxidized LDL (oxLDL). Here we report on the regulation of MS by an atherogenic high-fat diet and oxLDL, and on the inability of MS in transfected cells to bind oxLDL. MS expression was markedly increased in the livers of atherosclerosis-susceptible C57BL/6 and atherosclerosis-resistant C3H/HeJ mice fed an atherogenic high-fat diet. In resident-mouse peritoneal macrophages, treatment with oxLDL upregulated MS mRNA and protein expression 1.5- to 3-fold. MS, overexpressed in COS-7 cells through adenovirus mediated gene transfer, bound oxLDL by ligand blotting. However, no binding of oxLDL to MS was observed in intact transfected COS-7 and Chinese hamster ovary cells, despite significant cell surface expression of MS. Furthermore, inhibition of MS through gene silencing did not affect the binding of oxLDL to macrophages. We conclude that although MS expression in macrophages and Kupffer cells is responsive to a proatherogenic inflammatory diet and to oxLDL, MS does not function as an oxLDL receptor on the cell surface.**—de Beer, M. C., Z. Zhao, N. R. Webb, D. R. van der Westhuyzen, and W. J. S. de Villiers. **Lack of a direct role for macrosialin in oxidized LDL metabolism.** ***J. Lipid Res.*** **2003.** 44: **674–685.**

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There is accumulating evidence that oxidative modifications of LDL contribute to early atherogenesis (1). The uncontrolled uptake of modified and oxidized LDL (oxLDL) by vascular wall macrophages in the subendothelial space via scavenger receptors produces excessive lipoprotein-derived cholesteryl ester (CE) accumulations characteristic of foam cell formation. Macrophage-derived foam cells are the hallmark of fatty streaks and atherosclerotic plaques in human disease and in animal models of atherosclerosis. A number of different macrophage cell surface scavenger receptors for modified LDL have now been identified (2), and key questions are the relative contribution of the different macrophage scavenger receptors to atherogenesis and whether they play a protective role to clear oxidized lipids, as opposed to a pathological role in lesion development.

Data from studies with scavenger receptor class A (SR-A) gene knockout animals indicate that SR-A acts as a proatherogenic molecule in vivo (3, 4). This effect is modest, however, and requires backcrosses on atherosclerosis-susceptible apolipoprotein E (apoE)-deficient (±50% reduced relative to apoE$^{-/-}$) (3) or LDL receptor (LDLR) knockout animals (±20% reduced relative to LDLR$^{-/-}$) (4) to become apparent. There was no difference in the plasma clearance of acetylated LDL or oxLDL when SR-A-deficient animals were compared with wild-type animals (3). Recent studies have also shown that C57BL/6 mice lacking SR-A are protected from diet-induced atherosclerosis; furthermore, SR-A expression specifically in macrophages contributes significantly to lesion formation in C57BL/6 and LDLR null mice (5). These data support a role for SR-A in atherogenesis, but also indicate that macrophage scavenger receptors other than SR-A participate in the in vivo generation of foam cells.

CD36 is involved in the endocytosis of long-chain fatty acids, anionic phospholipids, and oxidized lipoproteins (6). CD36-null mice have increased fasting plasma cholesterol, and increased nonesterified free fatty acid and triacylglycerol levels. CD36-apoE double null mice have markedly decreased aortic lesions, both on normal and Western diet, when compared with controls, despite alterations in lipoproteins that correlate with increased atherogenicity (7, 8). Macrophages from CD36-apoE double knockout mice bound and internalized 60% less oxLDL. These findings indicate a major role for CD36 in murine atherosclerotic lesion development in vivo. In contrast,

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Abbreviations: CE, cholesteryl ester; oxLDL, oxidized low density lipoprotein; SR-A, scavenger receptor class A.

[1] To whom correspondence should be addressed.
e-mail: wdevil0@uky.edu

mice containing a targeted deletion for SR-BI, the other member of the class B scavenger receptor family, have dramatically accelerated atherosclerosis (9, 10). Hepatic overexpression of SR-BI suppresses atherosclerosis (11, 12), supporting a role for SR-BI as an antiatherogenic scavenger receptor. Although SR-BI is expressed on macrophages and binds oxLDL (13, 14) (in addition to its more clearly defined role as an HDL receptor), its relative importance as a macrophage oxLDL receptor is as yet unknown.

Murine macrosialin (MS) and its human homolog CD68 are extensively glycosylated transmembrane proteins expressed in macrophage and macrophage-related cells, including liver Kupffer cells (15). MS is a member of the lysosomal-associated membrane protein family of proteins and shares homology with their cytoplasmic tail and C-terminal domains (16). MS is predominantly a late endosomal protein but is also found on the cell surface (15, 17). Interest in MS as a scavenger and oxLDL receptor arose when it was suggested, on the basis of ligand blotting, to bind oxLDL (18, 19). Further evidence for a role for MS in modified LDL catabolism includes its identification in liver Kupffer cells as the major oxLDL binding protein (20), and its prominent expression in macrophages in atherosclerotic plaques from apoE knockout mice (21).

Considerable evidence now points to a key role of inflammatory processes in the pathogenesis of atherosclerosis (22). A high-fat, high-cholesterol atherogenic diet results in the accumulation of oxidized lipids in the livers and arteries of fatty streak susceptible C57BL/6 mice and in the induction of several inflammatory and oxidative stress responsive genes in the liver (23). We determined the effect of an atherogenic diet on the expression of oxLDL binding proteins, specifically MS as the major oxLDL binding protein, in the livers of different strains of mice. Using MS expression in transfected cell studies and in mice fed an atherogenic diet, as well as through inhibition by gene silencing, we further examined the in vitro and in vivo role of MS as an oxLDL receptor.

MATERIALS AND METHODS

Mice

C3H/HeJ, C57BL/6, and Swiss-Webster mice were purchased from Jackson Laboratories (Bar Harbor, ME). All of the mice were females 3–6 months old. The control diet was Purina chow (Ralston-Purina Co., St. Louis, MO) containing 4% fat. The atherogenic diets, obtained from Teklad (Madison, WI), contained 15.75% fat, 1.25% cholesterol, and 0.5% sodium cholate (cholate diet) (TD 90221), or 15.75% fat and 1.25% cholesterol, but no cholate (Western diet) (TD94059). All animals were maintained in a pathogen-free facility under equal light-dark cycles and with free access to water and food.

Cell culture

COS-7 cells were grown in DMEM supplemented with 10% FBS, and 1% penicillin and streptomycin. Chinese hamster ovary (CHO)-LDLA cells (clone 7, which lack the LDL receptor) (provided by M. Krieger) as well as cells stably transfected with MS were cultured in Ham's F12 medium containing 5% (v/v) FBS, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Resident peritoneal macrophages were harvested from female Swiss-Webster mice (age 6 weeks) by peritoneal lavage using ice-cold PBS. The cells were washed, counted, and seeded at 1×10^6 cells/24-well plate in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum. Nonadherent cells were removed after 60 min by three washes with RPMI medium only. The remaining cells were incubated in DMEM containing 2% LPDS with or without minimally modified LDL (MM-LDL) (50 μg/ml) and oxLDL (50 μg/ml). Murine macrophage-like cell lines RAW264.7 and J774.A1 were grown in DMEM containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL Technologies, Grand Island, NY).

Confocal laser-scanning fluorescence microscopy

COS-7 and RAW264.7 cells were grown on glass cover slips in 12-well plates, fixed in 3% paraformaldehyde, washed with PBS, blocked with 10% FBS, and incubated with the MS-specific monoclonal antibody FA-11. The secondary antibody was Cyanin Cy3-conjugated anti-rat IgG. Samples were analyzed in a Leica TCS NT/SP confocal laser microscope (Wetzlar, Germany).

LDL isolation, oxidation, and radiolabeling

LDL (d = 1.019–1.063 g/ml) was isolated from fresh normal human plasma by sequential ultracentrifugation, dialyzed against 150 mM NaCl, 0.01% EDTA (pH 7.4), sterile-filtered through 0.45 μm filters (Millipore, Bedford, MA), and stored under nitrogen at 4°C as previously described (24). Protein quantitation was by the method of Lowry et al. (25).

OxLDL used to stimulate MS expression in peritoneal macrophage was prepared as described (26) by incubation in Ham's F-10 medium containing 5 μM $CuSO_4$ for 24 h at 37°C, and MM-LDL was obtained by incubating LDL in PBS containing 2 μM $CuSO_4$ for 4 h at 37°C. These oxLDLs had relative electroporetic mobilities of 3.0 and 1.7, respectively. OxLDL used for COS cell experiments was prepared as described (27), had an average electrophoretic mobility of 1.3, and exhibited extensive aggregation of apoB as visualized by SDS-PAGE. Oxidation was terminated by the addition of 10 μM EDTA, and the modified LDL was dialyzed against 150 mM NaCl, 0.01% EDTA (pH 7.4), sterile-filtered through 0.45 μm filters (Millipore, Bedford, MA), and stored under nitrogen at 4°C.

Lipoproteins were iodinated by the iodine monochloride method (28). LDL was double-labeled by iodinating the protein component, and the cholesteryl ester was traced with nonhydrolyzable, intracellularly trapped [1α,2 α(n)^{3}H]cholesteryl oleyl ether. Briefly, [1α,2 α(n)^{3}H]cholesteryl oleyl ether suspended in dichloromethane was coated onto the inside of a glass tube by evaporation under nitrogen (29). Partially purified CETP was used to transfer the cholesterol ester to iodinated LDL. Double-labeled LDL was separated from CETP by ultracentrifugal flotation at a density of 1.063 g/ml. The integrity of all the labeled lipoprotein ligands was verified by SDS-PAGE and gradient gel electrophoresis, and the lipid compositions determined enzymatically (WAKO Chemicals, Osaka, Japan).

Lipid analyses

Mice were fasted 6 h prior to blood collection for plasma lipid analyses. Total cholesterol and triglycerides were determined enzymatically (WAKO Chemicals, Osaka, Japan and Sigma, St. Louis, MO). HDL cholesterol (HDL-C) was measured enzymatically after precipitation of LDL and VLDL by heparin and manganese (WAKO Chemicals, Osaka, Japan).

RNA analysis

Total RNA was isolated from the livers of mice using TRIzol™ reagent (Life Technologies, Grand Island, NY), and 20 μg ali-

quots were subjected to Northern blot hybridization analysis as described (30). DNA fragments used as probes were random labeled with [α-^{32}P]dCTP (ICN Pharmaceuticals, Inc., CA). MS mRNA was identified with an N terminal *Eco*RI fragment (~700 bp) obtained from MS cDNA (31). Macrophages present in the liver (both resident Kupffer cells and infiltrating macrophages) were probed for with a 700 bp *Cla*I-*Apa*I restriction fragment obtained from the cDNA for the macrophage-specific membrane protein F4/80 (32). SR-BI mRNA was identified with a ~1 kb *Pst* fragment excised from the cDNA (33), and SR-A mRNA was identified with a 236 bp fragment generated by PCR (34). To verify the quantity of RNA loaded on gels, Northern blots were hybridized simultaneously with a *Hind*III fragment of the cDNA encoding the constitutive 18*S* ribosomal protein L30 (33).

For PCR analysis, total RNA was isolated from cultured macrophages as above, and cDNA was generated by reverse transcriptase using AMV reverse transcriptase (Roche Diagnostics, Basel, Switzerland). PCR amplification was performed with *a*) MS-specific primers (5′ primer: 5′-CTAGCTGGTCTGAGCATCTCT-3′; 3′ primer: 5′-TTCCACCGCCATGTAGTCC-3′; size of amplified fragment: 355 bp); and *b*) murine β-actin specific primers (5′primer: 5′-TCAGAAGGACTCCTATGTGG-3′; 3′ primer: 5′-GTACGACCAGAGGCATACAG-3′; size of amplified fragment: 299 bp). The PCR products were subjected to gel electrophoresis and the bands scanned by densitometry. The signal of the MS-specific band was compared with that of the β-actin-specific band generated under the same conditions. Differences in intensity of PCR bands were confirmed by 10-fold serial dilutions of cDNA samples to ensure linearity of the amplification reaction. The results discussed were representative of three independent experiments.

Inhibition of MS expression in cell lines

The expression of MS was inhibited by gene silencing through targeted mRNA degradation using a Silencer™ siRNA Construction Kit according to the manufacturer's instructions (Ambion Inc.). The MS-specific sense oligonucleotide 5′AAGACAGTCTTCCTCTGTTCCCCTGTCTC was designed according to sequences 46 bp downstream of the AUG start codon. Transfection of the dsRNA was achieved using a GeneSilencer™ siRNA Transfection Reagent from Gene Therapy Systems, Inc. according to the manufacturer's instructions.

Western blot analysis

Membranes were prepared from mouse livers as described (33), and 10 μg aliquots were subjected to Western blot analyses using nonreducing SDS-PAGE. Samples were transferred onto nitrocellulose membrane and incubated with the MS-specific monoclonal antibody FA-11 (35), anti-mCD36 (27), or anti-mSR-BI antibodies (33), followed by chemiluminescent detection (ECL, Amersham Biosciences, Piscataway, NJ) and autoradiography. Cultured cells were lysed in 25 mM morpholinoethanesulfonic acid (pH 6.5), 150 mM NaCl, 1% Triton-X-100, and 60 mM octylglucoside. After incubation on ice for 10 min, the cell lysates were spun at 13,000 *g* for 10 min, and 10 μg aliquots of the supernatant were subjected to Western analysis as described above. Films were analyzed by densitometric scanning (Molecular Dynamics, Sunnyvale CA). There was a near-linear relation between optical density and amount of protein over the range of 0 μg to 100 μg of macrophage protein lysate loaded.

Ligand blots

OxLDL binding to MS was demonstrated by ligand blots as described (20). Briefly, 50–100 μg of solubilized cell protein was transferred onto nitrocellulose membrane. After blocking for 1 h, the blots were incubated with oxLDL at a concentration of 10 μg/ml for 2 h and washed extensively to remove unbound material. The bound oxLDL was visualized with an anti-apoB antibody (Calbiochem, Darmstadt, Germany) using a peroxidase-labeled secondary antibody and ECL detection.

Preparation of adenoviral vectors

Sequences encoding mouse MS, mCD36, or mSR-BI were inserted into an adenovirus expression vector pAdCMV.link (27, 33, 36) containing the cytomegalovirus immediate-early enhancer promoter element to yield pAdMS, pAdCD36, or pAdSR-BI. The expression plasmids were cotransfected into 293 cells with replication-defective adenoviral DNA as described previously (33) to generate recombinant adenoviruses expressing MS, mCD36, or mSR-BI. Adnull (provided by Dr. D. J. Rader) is a recombinant virus with analogous adenoviral sequences containing no transgene.

Flow cytometry

Cells were stained with primary and FITC-conjugated secondary antibodies before flow cytometry (FACScan®, Becton-Dickinson and Co., Mountain View, CA). Cells and debris having low-forward scatter were routinely gated out of the analysis. For visualization of intracellular antigens, cells were permeabilized with 1% (wt/vol) saponin in PBS with 1% FCS and 1% normal mouse serum (NMS) for 30 min. All subsequent steps were performed in the presence of 0.1% (wt/vol) saponin in PBS with 1% FCS and 1% NMS (34).

Biotinylation and immunoprecipitation of cell proteins

The total cellular or surface expression of MS in COS cells infected by AdMS was determined by biotinylation and immunoprecipitation essentially as described (19). Briefly, proteins on the surface of intact COS infected with AdMS or a control Adnull virus (which does not express any protein) were biotinylated with EZ-link™ Sulfo-NHS-LC-Biotin (Pierce Chemical Co.) at 100 μg/ml for 1 h at 4°C. After washing, the cells were precipitated at 300 *g*. The insoluble material from octylglucoside cell extracts were sedimented at 100,000 *g* and the MS in the supernatant immunoprecipitated with a protein G immunoprecipitation kit (Pierce Chemical Co.) according to the manufacturer's instructions using the MS-specific monoclonal antibody (MAb) FA-11. Total cellular MS was determined by biotinylating octylglucoside cell extracts with EZ-link™ Sulfo-NHS-LC-Biotin (Pierce Chemical Co.) at 4 mg/ml for 1 h at 4°C, and immunoprecipitation of the soluble material as above after sedimention at 100,000 *g*. Biotinylated proteins were visualized by Western blot using streptavidin conjugated to alkaline phosphatase (Novagen) at a 1:5,000 dilution.

Adenoviral vector treatments and analysis of plasma lipids

Male C57BL/6 mice weighing at least 25 g were injected in the tail vein with 5×10^{10} particles of recombinant adenoviruses Adnull, AdMS, or AdSR-BI in 100 μl of PBS. Plasma samples were collected from mice fasted for 10 h prior to collection. Plasma lipids were measured enzymatically as described above.

Ligand binding and uptake assays

COS-7 and CHO cells were seeded in 12-well plates 48 h prior to assays (2.5×10^5 cells per well). Preliminary experiments with adenovirus expressing green fluorescent protein (GFP) were performed to determine the viral dose to achieve gene transfer in more than 95% of treated cells. This level of expression was confirmed for AdCD36 and AdMS by indirect immunofluorescence. Adenoviral vector-mediated gene overexpression was performed by addition of Adnull, AdCD36, or AdMS at a viral dose of 1,000–4,000 particles [multiplicity of infection (MOI) of 10 to

40 plaque forming units (pfu)] per cell 24 h prior to assay. COS-7 cell association assays, described previously (27), were performed at 4°C or 37°C in DMEM supplemented with 0.5% essentially fatty acid free BSA, 1% penicillin and streptomycin, and radiolabeled lipoprotein. CHO-cell association assays were performed at 37°C in Ham's F-12 containing 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 0.5% essentially fatty acid free BSA, and radiolabeled lipoprotein (33). After incubating for the indicated times, unbound ligand was removed from cells by washing four times with 50 mM Tris-HCl and 150 mM NaCl (pH 7.4) containing 2 mg/ml fatty acid free BSA, followed by two washes in the same buffer without BSA. All washes were performed at 4°C with prechilled solutions. Noniodide trichloroacetic acid-soluble degradation products were measured in the media. Cells were solubilized in 0.1 N NaOH for 60 min at room temperature prior to protein and radioactivity quantitation. Receptor-specific cell association values were calculated as the difference between the total for AdCD36 or AdMS-expressing cells and corresponding values for Adnull control cells. K_d values were determined by nonlinear regression analysis of receptor-specific cell association values using Prism® software (GraphPad Software, San Diego, CA).

Statistics

Data on comparisons of mRNA and protein levels were expressed as mean ± SD. Results were analyzed by Student's *t*-test. A value of $P < 0.05$ was considered significant.

RESULTS

High-fat atherogenic diet increases hepatic MS mRNA and protein expression selectively

We compared the effect of two high-fat diets, the so-called Western diet and cholate diet, on the expression of MS by feeding atherosclerosis-susceptible C57BL/6 mice (six animals per group) the high fat diets for 1 week. Northern blot analysis of the livers of these animals using a MS-specific probe showed prominent upregulation of this molecule by both diets, but no difference in response to the two diets (**Fig. 1A**). Western blot analysis of MS protein expression in the livers mimicked these results (data not shown). In view of this result, we explored the effect of an atherogenic diet containing 15.75% fat, 1.25% cholesterol, and 0.5% sodium cholate on the presence of MS and other oxLDL binding proteins in the liver by feeding C57BL/6 mice (four animals per group) this diet for periods varying from 3 days to 5 weeks. Northern blot analysis of the livers of these animals was performed using receptor-specific probes as indicated in Materials and Methods (Fig. 1B). The relevant bands on the autoradiographs were quantified by densitometric scanning and compared with the mRNA encoding the constitutive 18*S* ribosomal protein, L30 (Fig. 1C). The constitutively expressed macrophage-specific membrane molecule, F4/80, which has no known lipid-related function, was used as a marker for macrophage presence and infiltration into the liver during the treatment course (37).

Prominent MS mRNA upregulation was already observed within 3 days and increased up to 10.5-fold after 5 weeks of atherogenic diet. A lesser 3.2-fold increase in F4/80 mRNA expression indicated that increased MS mRNA levels were due to selective upregulation in hepatic macrophages, most likely in resident Kupffer cells, but also in macrophages that had infiltrated into the liver. This finding was confirmed by immunohistochemical studies with the FA-11 and F4/80 MAbs (data not shown). SR-A mRNA increased slightly after 5 weeks of dietary treatment, which could be accounted for by the increased macrophage infiltration. Neither CD36 nor SR-BI expression (data not shown) responded to the high-fat diet.

Using a standard protocol, tissue lysates were prepared from the livers of animals fed an atherogenic diet. Western blot analysis using the MS-specific antibody FA-11 (15) is depicted in **Fig. 2**. Increased MS protein expression is evident as early as 3 days following initiation of the high-fat diet and continues to increase in line with the increased mRNA levels observed. These data indicate that the regulation of MS expression occurs at the transcriptional level. MS protein also showed significant size differences on SDS-PAGE analysis with increasing exposure to the high-fat diet. These changes are consistent with alterations of glycosylation that have been described to occur in the mucin-like extracellular domain of MS in response to inflammatory stimuli (15, 38).

The effects of high-fat diet on MS expression in atherosclerosis-susceptible and atherosclerosis-resistant mice

We subsequently compared the induction of MS in the livers of atherosclerosis-susceptible C57BL/6 mice and atherosclerosis-resistant C3H/HeJ mice fed an atherogenic high-fat diet for a period of 7 weeks (39). The expression of MS and the macrophage-specific marker F4/80 mRNA levels were normalized to that of the mRNA encoding the constitutive 18*S* ribosomal protein, L30 (**Fig. 3**).

MS mRNA upregulation was observed in both mouse strains, but to a lesser extent in the atherosclerosis-resistant C3H/HeJ (4-fold) compared with the atherosclerosis-susceptible C57BL/6 animals (6-fold). Hepatic macrophage infiltration (as measured by the F4/80 signal) was also more prominent in the susceptible strain (3-fold increase) than in the resistant strain (1.6-fold). However, the MS to F4/80 ratio was greater in the atherosclerosis-resistant C3H/HeJ strain (2.5) than in the atherosclerosis-susceptible C57BL/6 (2.0) mice. In addition to the differences in levels of macrophage infiltration into the liver, this relatively more selective upregulation may indicate a protective role for MS in the atherosclerosis-resistant C3H/HeJ strain.

Oxidatively modified LDL increases MS expression in mouse peritoneal macrophages

The high-fat cholic acid diet is known to impart an inflammatory proatherogenic stimulus that enhances lipoprotein oxidation in vivo (23, 40). OxLDL is a potential candidate responsible for inducing MS expression in view of its oxLDL binding ability. To determine the effects of modified lipoproteins on MS expression in primary cells, we treated resident peritoneal macrophages with MM-LDL and oxLDL for 48 h at 37°C. RT-PCR analysis was performed using specific primers for MS and β-actin. Western

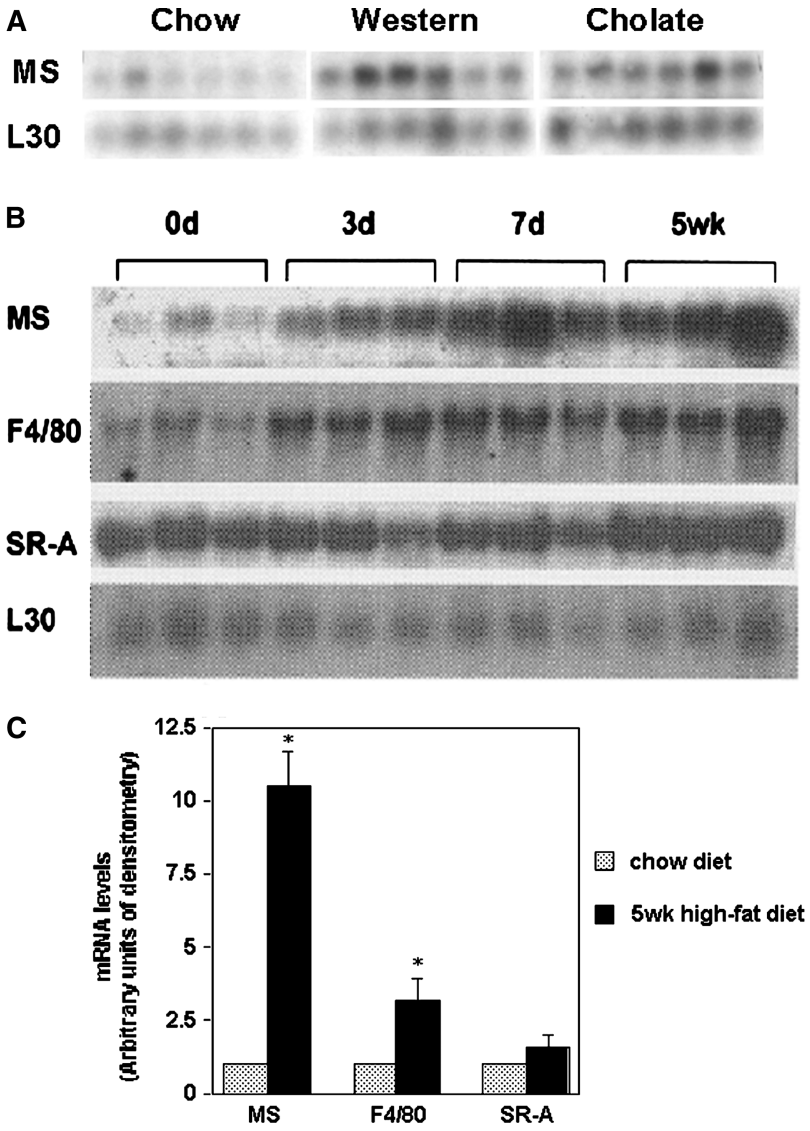

Fig. 1. A high-fat diet increases hepatic macrosialin (MS) mRNA expression selectively. Atherosclerosis-susceptible C57BL/6 mice were fed an atherogenic diet containing 15.75% fat and 1.25% cholesterol (Western diet), or a diet containing 15.75% fat, 1.25% cholesterol, and 0.5% sodium cholate (cholate diet) for periods varying from 3 days to 5 weeks. A: Total RNA was isolated from the livers of mice fed a Western diet or cholate diet for 1 week (six mice per group), and 20 μg aliquots were subjected to Northern blot analysis using a MS-specific probe. A cDNA probe for the constitutively expressed 18*S* ribosomal protein L30 was used as a measure of RNA quality and quantity. B: Total RNA was isolated from the livers of three mice fed a cholate diet at each time point using a standard protocol, and 10 μg aliquots were subjected to Northern blot analysis using the receptor-specific probes indicated. A cDNA probe for the constitutively expressed 18*S* ribosomal protein L30 was used as a measure of RNA quality and quantity. C: Quantitation of MS upregulation in four mice after 5 weeks of high-fat diet. The relevant bands on the autoradiographs were quantified by densitometric scanning and normalized to L30 expression. The macrophage-specific membrane protein, F4/80, which has no known lipid-related function, was used as a marker for macrophage presence and infiltration into the liver. Data shown are mean values ± SD. Differences related to chow diet were analyzed by Student's *t*-test; $*P < 0.05$.

blot analysis was performed using MS-specific antibody FA-11 (**Fig. 4**), and the blots were scanned densitometrically. MM-LDL and oxLDL both significantly increased MS mRNA (1.5- to 3-fold) (data not shown) and protein expression in murine resident peritoneal macrophages. OxLDL had a more marked effect (2.5-fold) than MM-LDL (1.8-fold). This confirms previous work done by Steinberg and colleagues (26).

Functional comparison of MS and CD36 expressed by adenovirus

Macrophages express other potential oxLDL binding proteins such as CD36, SR-A, and SR-BI that may confound the interpretation of oxLDL interaction with macrophages. To diminish the role of competing oxLDL binding proteins, MS was expressed by adenoviral-mediated gene transfer in COS-7 cells. As shown in **Fig. 5**, MS was reproducibly

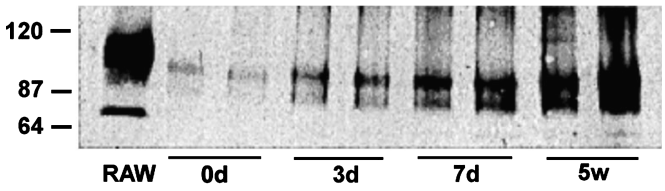

Fig. 2. High-fat diet increases hepatic MS protein expression. Membranes were prepared at 4°C from the livers of C57BL/6 mice fed an atherogenic high-fat diet for the indicated time periods. Detergent extracts (10 μg) were subjected to SDS-PAGE in 5–20% polyacrylamide gradient gels, and the proteins were electrotransferred to nitrocellulose membranes. Western blot analysis was performed using the MS-specific monoclonal antibody (MAb) FA-11. RAW = 10 μg lysate prepared from RAW264.7 murine macrophage-like cell line as a positive control.

expressed at high concentrations in COS-7 cells using a second-generation adenoviral system as described in Materials and Methods (33). Suitable titration of the viral dose resulted in greater than 95% cellular expression, as determined by indirect immunofluorescence using a rhodamine-labeled anti-rat IgG antibody to FA-11. No expression of MS was observed in COS-7 cells transfected with a control virus (Adnull) that expresses no protein. The size of the expressed MS protein in COS-7 cells is somewhat smaller than that seen in murine macrophage cell lines (Fig. 5). This is likely due to differences in glycosylation. Overexpression of MS by adenoviral-mediated gene transfer in the macrophage cell lines J774.A1 and RAW264.7 resulted in only modest increases above basal expression.

MS is a macrophage-specific late endosomal protein, and its predominant intracellular localization may argue against a functional role as an oxLDL receptor expressed on the plasma membrane. The cellular distribution (cell surface versus intracellular) of MS was examined by means of flow cytometry, immunofluorescence and biotinylation, and immunoprecipitation in intact and permeabilized COS-7 cells treated with AdMS. The data generated by flow cytometry (**Table 1**) show that significant levels of surface expression, ranging from 37–51% of total expression,

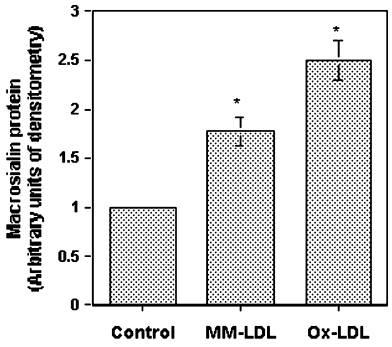

Fig. 4. Oxidatively-modified LDL increases MS expression in peritoneal macrophages. Resident murine peritoneal macrophages were treated with minimally modified LDL (50 μg/ml) and oxidized LDL (oxLDL) (50 μg/ml) for 48 h at 37°C. Western immunoblots were done using the MS-specific MAb FA-11 and scanned densitometrically. The results shown are representative of three independent experiments. Data shown are normalized to values in untreated cells and are mean values ± SD. Differences related to treatments were analyzed by Student's *t*-test; $*P < 0.05$.

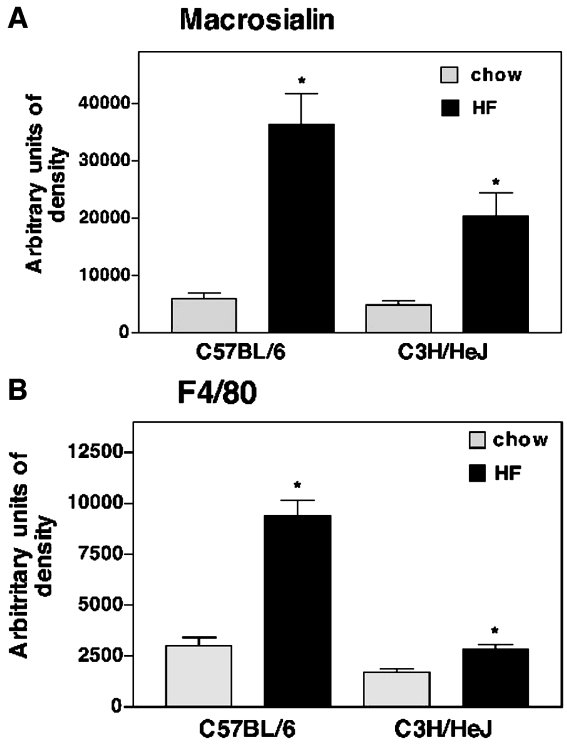

Fig. 3. The effects of high-fat diet on MS expression in atherosclerosis-susceptible and atherosclerosis-resistant mice. Atherosclerosis-susceptible C57BL/6 mice (n = 4) as well as atherosclerosis-resistant C3H/HeJ mice (n = 4) were fed an atherogenic high-fat diet for 7 weeks. Total RNA was isolated from mouse livers using a standard protocol and aliquots (10 μg) subjected to Northern blot analysis. The relevant bands on the autoradiographs were quantified by densitometric scanning and normalized for mRNA of L30, a constitutive 18*S* ribosomal molecule. The macrophage-specific membrane protein, F4/80, was used as a marker for macrophage presence and infiltration into the liver. A: MS expression in atherosclerosis-susceptible C57BL/6 and atherosclerosis-resistant C3H/HeJ mice. B: F4/80 expression in atherosclerosis-susceptible C57BL/6 and atherosclerosis-resistant C3H/HeJ mice. Data shown are mean values ± SD. Differences related to diets and mouse strains were analyzed by Student's *t*-test; $*P < 0.05$.

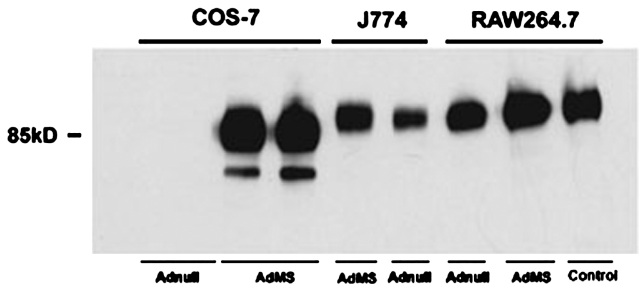

Fig. 5. Adenoviral-mediated overexpression of MS in COS-7 cells and macrophage-like cell lines. MS expression in COS-7, J774.A1, and RAW 264.7 cells transfected with Adnull or AdMS were analyzed by immunoblot analysis. COS-7 cells were treated with a multiplicity of infection (MOI) of 40 pfu per cell, and J774.1 and RAW 264.7 cells with a MOI of 80 pfu per cell. RAW 264.7 cell lysate served as a positive control. Aliquots of 10 μg cell lysate were loaded per lane.

TABLE 1. Flow cytometry of AdMS expression in intact and permeabilized COS-7 cells

AdMS MOI	Intact (Surface)	Permeabilized (Internal)	Surface/Surface + Internal
	fluorescence intensities		%
0 (control)	0	0	0
20	62 ± 8	59 ± 5	51
40	76 ± 9	104 ± 9	42
80	117 ± 13	198 ± 15	37

The surface and intracellular distribution of macrosialin (MS) expressed in AdMS-treated COS-7 cells were examined by flow cytometry. Cells were treated in vitro for 48 h with various multiplicities of infection of AdMS as indicated and stained with monoclonal antibody (MAb) FA-11 or IgG2a isotype, and FITC-conjugated secondary antibody before flow cytometry. For visualization of intracellular MS, cells were permeabilized with 1% (wt/vol) saponin in PBS. Results show the mean values ± SD (single experiment in triplicate) of specific fluorescence intensities determined by subtracting geometric mean fluorescence obtained with the isotype control from that obtained with the FA-11 MAb. Cell surface expression (intact cells) was quantified as a percentage of the total cellular expression (intact plus permeabilized values).

could be achieved in COS-7 cells following administration of AdMS at various titers. This level of surface expression is much higher than the less than 5% cell surface expression described for intact RAW264.7 cells (15, 19).

Confocal immunofluorescense microscopy using the MS-specific MAb FA-11 (**Fig. 6A**) showed no expression of MS in intact or permeabilized COS cells treated with a control Adnull virus at a viral dose of 4,000 particles MOI of 40 pfu per cell 24 h prior to assay (Fig. 6AA, AB). Although permeabilized RAW cells contained considerable levels of MS (Fig. 6AD), very little MS was present on the surface of intact RAW cells (Fig. 6AC). In COS cells treated with AdMS at a MOI of 40 pfu, the surface expression of MS on intact cells represented a significant proportion of the total cellular expression present in permeabilized cells (Fig. 6AE, AF).

Surface expression of MS in COS cells infected with AdMS at a MOI of 40 pfu was also verified by biotinylation of surface proteins followed by immunoprecipitation of cell membrane extracts with the MS-specific MAb FA-11 (Fig. 6B). For comparison, the total cellular content of MS was assessed by the biotinylation of whole cell extracts followed by immunoprecipitation. Densitometric scanning utilizing a standard curve generated from serial dilutions of total cellular MS (not shown) indicated that the cell surface expression (lanes 2 and 3) comprised ~60% of the total cellular content of MS (lane 1). No MS was present on the surface of cells treated with a control Adnull virus (lane 4). For these reasons, further functional studies were done in COS-7 cells overexpressing MS.

Ligand blots with oxLDL were performed to determine whether MS expressed in COS-7 cells has the ability to bind oxLDL. We first determined and confirmed previous reports (20), using an antibody directed against apoB, that oxLDL binds to liver cell extracts. The band obtained corresponds in size to the MS-specific band detected by Western blotting using the MS-specific antibody FA-11 (**Fig. 7A**). This indicates that MS, expressed by Kupffer

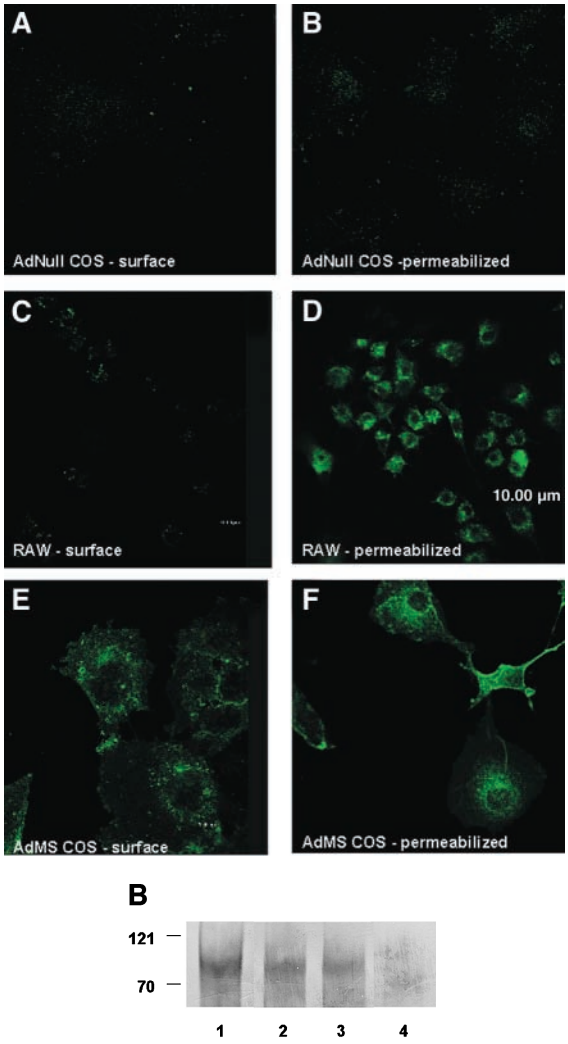

Fig. 6. MS distribution in intact and permeabilized COS-7 cells. A: The cellular distribution of MS expressed by adenoviral vector in COS-7 cells was determined by immunofluorescence confocal microscopy using the MS-specific MAb and a Cyanin Cy3-conjugated secondary antibody. Surface expression in intact cells is depicted in the left panel and the total expression present in permeabilized cells is shown on the right. COS cells were treated with a control Adnull virus at a MOI of 40 pfu (top panel) or AdMS at a similar MOI (bottom panel). The macrophage-like cell line RAW 264.7 is depicted in the middle panel for comparison. B: COS cells treated with AdMS at a MOI of 40 pfu were subjected to cell-surface specific biotinylation and immunoprecipitation of the detergent extracts with the MS-specific MAb FA-11, as described in Materials and Methods (Lanes 2 and 3). Total cellular MS was determined by biotinylation of whole-cell extracts followed by immunoprecipitation (Lane 1). The biotinylation of whole-cell extracts from COS cells treated with control Adnull at an MOI of 40 pfu is shown in Lane 4.

cells, is the oxLDL binding protein. Ligand blots, utilizing the same system, showed that MS in transfected COS-7 cell extracts was able to bind oxLDL, in contrast to COS-7 cells treated with Adnull (Fig. 7B). Western blot size comparison using the FA-11 antibody suggests that this property is conferred by MS.

The functional activity of MS in intact cells was compared with that of mCD36, a well-characterized receptor for oxLDL. The concentration-dependent binding of ^{125}I-

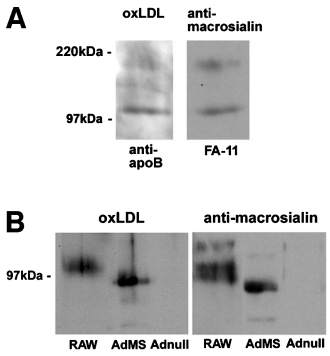

Fig. 7. OxLDL ligand blots of liver cells and COS-7 cells expressing MS. A: Liver-cell lysate (50 μg) was subjected to ligand blot analysis as described in Materials and Methods, and the bound oxLDL was visualized with an anti-apolipoprotein B (apoB) antibody. Size comparison of a Western blot of the same liver lysates (10 μg) with the MS-specific MAb FA-11 suggests binding of oxLDL to MS expressed on Kupffer cells. B: Lysates (100 μg) from COS-7 cells treated with AdMS or Adnull, and RAW 264.7 cells lysates, were subjected to ligand-blot analysis. Bound oxLDL was visualized with an anti-apoB antibody. Western blot analysis of the same cell lysates (10 μg) using the MS-specific MAb FA-11 suggests binding of oxLDL to MS.

labeled oxLDL at 4°C was examined in COS-7 cells treated with control the Adnull, AdMS, or AdCD36 virus (**Fig. 8**). Cell-associated radioactive ligand was measured as previously described (27, 33). The binding of ^{125}I-oxLDL to cells treated with AdCD36 increased in a dose-dependent and saturable manner. The CD36-specific binding at 4°C showed a B_{max} of 1,338 ng/mg cell protein and a dissociation constant (K_d) of 4.8 ± 1.3 μg/ml, which is similar to values reported by us and others for oxLDL binding to mCD36 (27, 41). Binding to control Adnull transfected cells reflects a lower affinity binding site (K_d 40.4 μg/ml). In contrast to mCD36, no MS-specific binding of oxLDL was observed, in spite of ample expression of MS (Fig. 8). These data indicate that MS does not serve as a receptor for oxLDL on the surface of intact cells.

We also examined the interaction of ^{125}I-labeled oxLDL with MS and mCD36 treated COS-7 cells at 37°C (Fig. 8). Cell-associated and noniodide TCA-soluble degraded radioactive materials were measured as previously described (27). The data depict MS- and CD36-specific values that were calculated as the difference between cells treated with AdCD36 or AdMS, and cells treated with control Adnull virus. At a concentration of 10 μg/ml, there was a time-dependent increase in the association of ^{125}I-labeled oxLDL to CD36, with small amounts of ligand degradation (degradation products <10% of cell-associated ligand). This reflects predominant binding to mCD36 expressed at the cell surface with little internalization of the ligand. In contrast to CD36, very little MS-specific cell association and degradation of ^{125}I-labeled oxLDL occurred, in spite of significant protein expression. Extended incubations for up to 16 h did not result in increased association or increased degradation of oxLDL in MS-expressing cells (data not shown). In a number of experiments, we were also unable to show any differences in oxLDL association or degrada-

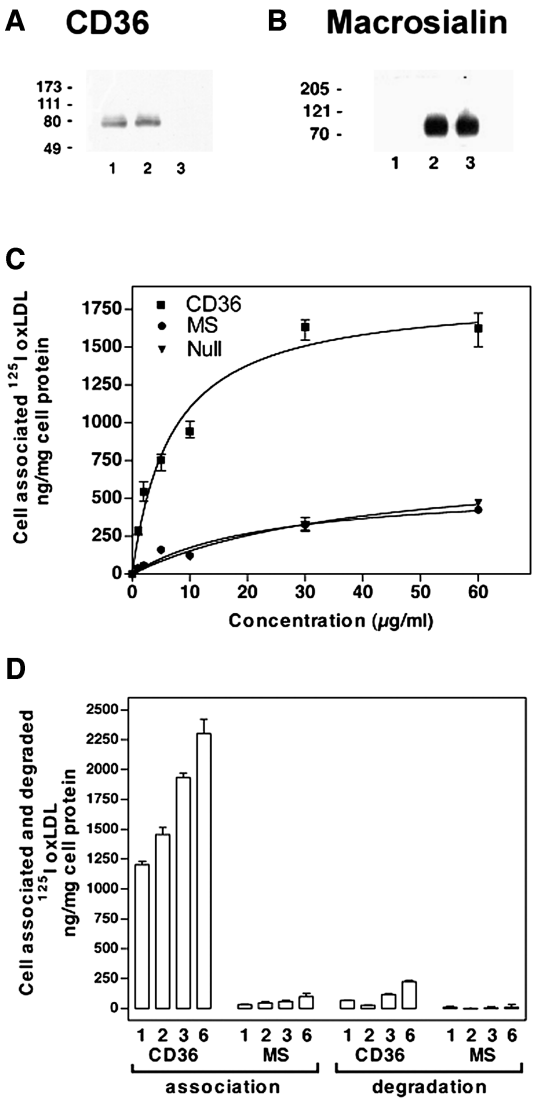

Fig. 8. Association of oxLDL with COS-7 cells expressing MS and CD36 by adenoviral vector. A: Immunoblot analysis of COS-7 cells treated with AdCD36 or control Adnull virus. Cells were exposed to an MOI of 10 pfu per cell, harvested 24 h later, and analyzed. Each lane contains 5 μg of cell protein. Lanes 1 and 2, AdCD36; Lane 3, Adnull. B: Immunoblot analysis of COS-7 cells treated with AdMS or control Adnull virus at an MOI of 40 pfu per cell. Cells were harvested 24 h later and analyzed. Each lane contains 5 μg of cell protein. Lane 1, Adnull; Lanes 2 and 3, AdMS. C: Dose-dependent binding of human ^{125}I-labeled oxLDL to COS-7 cells treated with AdCD36 (10 MOI), AdMS (40 MOI), or control Adnull virus (10 and 40 MOI, respectively). COS-7 cells were incubated with increasing concentrations of ^{125}I-labeled human oxLDL at 4°C for 2 h. The cell-associated label was quantitated as described in Materials and Methods. Shown are total cell-associated values. The data represent mean values ± SD from triplicate determinations. Similar results were obtained in a second experiment with a different batch of oxLDL. D: Time-dependent association of human ^{125}I-labeled oxLDL with COS-7 cells treated with AdMS (40 MOI), AdCD36 (10 MOI), or control Adnull virus (40 and 10 MOI respectively), and incubated at 37°C for 1 h to 6 h, as indicated. The cell-associated label and degradation products in the medium were quantitated as described in Materials and Methods. MS- and CD36-specific values are shown that were calculated as the difference between AdMS- and AdCD36-treated cells and cells treated with Adnull. The data represent mean values ± SD from triplicate determinations. Similar results were obtained in two additional experiments performed with different batches of oxLDL.

tion by macrophage-like cell lines (RAW264.7 and J774.A1) overexpressing MS by adenoviral-mediated gene transfer (AdMS) compared with cells treated with control virus (Adnull) (data not shown).

To evaluate whether MS could be involved in the selective uptake of CE from oxLDL, analogous to the selective uptake of HDL-CE by SR-BI, the association of ^{125}I and [^{3}H]CE double-labeled oxLDL with MS was studied in CHO-MS and untransfected CHO-LDLA7 cells. As with COS-7 cells, we found no difference in the binding and degradation of oxLDL by CHO-MS and control CHO-LDLA7 cells, and no evidence of selective uptake of CE (data not shown).

We wished to examine the in vivo effect of MS overexpression on plasma lipids. Because MS expressed in hepatic parenchymal cells following adenoviral administration might have an altered function compared with that expressed in hepatic macrophages, the adenoviral approach was combined with upregulation of MS in hepatic macrophages by ingestion of a high-fat proinflammatory atherogenic diet. C57BL/6 animals were injected with 5×10^{10} particles of AdMS or control Adnull virus and placed on a high-fat cholic acid diet 1 day after virus injection. Immunoblot analysis of liver samples collected 8 days after adenoviral vector administration confirmed a 15-fold increase in MS expression (data not shown). Although 1 week of high-fat feeding resulted in elevated total cholesterol and lowered triglyceride levels, no differences were observed between the plasma lipids of high-fat fed mice injected with AdMS and control Adnull virus or mice fed a high-fat diet alone (data not shown). In contrast, administration of AdSR-BI resulted in a dramatic drop in total and HDL cholesterol (HDL-C) levels (data not shown). We therefore conclude that MS overexpressed in the liver by adenovirus or induced by a high-fat diet does not influence plasma lipid values.

Diminished expression of MS in macrophages does not alter oxLDL binding

The technique of gene silencing by MS-specific dsRNA was employed to inhibit the expression of MS in macrophages (42). Densitometric scanning of Western blots utilizing a standard curve generated by serial dilutions of control cells treated with a nonspecific RNA indicated that, in the macrophage-like cell line RAW 264.7, the expression of of MS was diminished to 37% of the expression in control cells treated with a nonspecific dsRNA (**Fig. 9A**). In resident peritoneal macrophages, MS expression was reduced to 64% of the expression in control cells. The association of 10 μg/ml of ^{125}I-labeled oxLDL for 4 h at 37° to these cells is depicted in Fig. 9B. No difference was observed in the binding of oxLDL to control RAW264.7 cells or those cells treated with either a MS-specific oligonucleotide or a scrambled oligonucleotide. A similar result was observed with resident peritoneal macrophages. The degradation of oxLDL by macrophages reached levels equivalent to the cell-associated levels and was higher than that observed in COS cells treated with AdMS (Fig. 8); however, the values for cells treated with a MS-specific oligonucleotide were no different from the values for those cells treated with a scrambled oligonucleotide. We therefore conclude that diminished MS expression does not alter oxLDL binding in macrophages or macrophage-like cell lines.

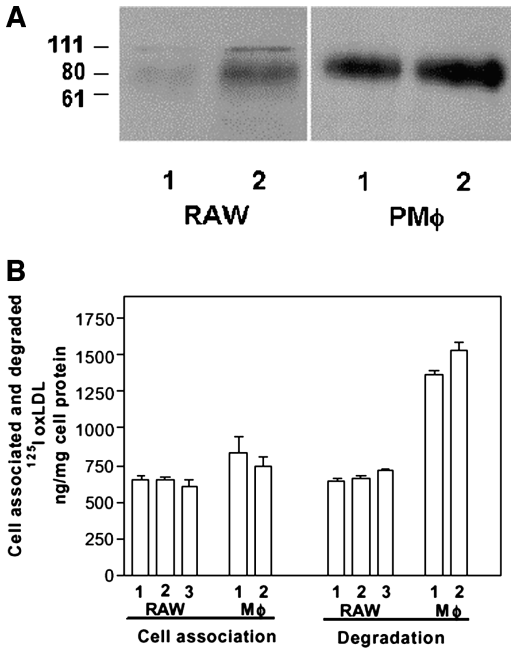

Fig. 9. Inhibition of MS expression by RNA silencing in macrophages and macrophage-like cell lines does not reduce binding of oxLDL. The technique of gene silencing described in Materials and Methods was employed to inhibit the expression of MS in RAW264.7 cells and in resident peritoneal macrophages. A: Western blot (15 μg total-cell lysate per lane) of MS expression in the macrophage-like cell line RAW264.7 and in resident peritoneal macrophages. Lane 1, cells treated with a MS-specific dsRNA; Lane 2, control cells treated with a nonspecific dsRNA. B: Binding of human ^{125}I-labeled oxLDL to macrophage-like RAW264.7 cells and to resident peritoneal macrophages. Lane 1, cells treated with a MS-specific dsRNA; Lane 2, cells treated with a nonspecific dsRNA; Lane 3, untreated RAW264.7 cells. Cells were incubated with 10 μg/ml of ^{125}I-labeled human oxLDL at 37°C for 4 h. The cell-associated label was quantified as described in Materials and Methods. Shown are total cell-associated values. The data represent mean values ± SD from triplicate determinations. Similar results were obtained in a second experiment with a different batch of oxLDL.

DISCUSSION

In this study, we examined the regulation of MS and its possible role as a receptor for oxLDL. We first investigated the regulation of MS by an atherogenic diet and modified lipoproteins. The data indicate the following: *i*) a high-fat, proinflammatory atherogenic diet, whether it contains cholate or not, causes significant specific upregulation of MS mRNA and protein expression in the livers of both atherosclerosis-susceptible and atherosclerosis-resistant mouse strains; and *ii*) oxidatively-modified LDL increased MS mRNA and protein expression 1.5- to 3-fold in resident murine peritoneal macrophages. In the second part of the study, we directly compared MS with the well-recognized

oxLDL receptor, murine CD36, for their ability to serve as oxLDL receptors using the same adenoviral vector expression system. The results indicate that MS, despite significant cell-surface expression, and in contrast with mCD36, show no evidence of oxLDL binding and/or internalization. Further studies in macrophages and macrophage-like cell lines similarly indicate that inhibition of MS through gene silencing does not alter the binding of oxLDL.

MS expressed on Kupffer cells has previously been shown by ligand blotting to be the major oxLDL binding protein in rodent liver (20). We show convincingly that a proinflammatory, atherogenic high-fat diet markedly upregulates MS mRNA and protein expression in liver macrophages in a specific manner independent of the atherosclerosis-susceptibility of the underlying mouse strain (C3H/HeJ or C57BL/6). The atherogenic diets used can cause the accumulation of oxidized lipids in the liver, activation of NF-κB, expression of oxidative stress responsive genes, and an inflammatory response (23). Interestingly, atherosclerosis-resistant C3H/HeJ mice showed much less hepatic macrophage infiltration (as measured by F4/80 signal).

Although the precise role of MS in atherogenesis remains unclear, we postulated that its upregulation in liver macrophages in response to high-fat diets may indicate a compensatory protective role. MS expression in the late endosomes of hepatic macrophages may be induced as part of the physiological inflammatory response in the scavenging or phagocytosis of noxious aggregated modified lipoproteins. Other well-characterized macrophage oxLDL binding scavenger receptors, such as SR-A, CD36, or SR-BI, did not markedly respond to the atherogenic diet. A 2 week high-cholesterol diet has previously been shown to regulate SR-BI differentially in rat liver, resulting in lowered SR-BI expression in parenchymal cells and increased expression in Kupffer cells (43). In another study, dietary polyunsaturated fatty acids, in contrast to saturated fatty acids, upregulated hepatic SR-BI expression in the hamster (44). The lack of dietary SR-BI response in our experiments might be explained by differences in diets, experimental methods (total liver extracts versus cell isolation), and species specificity.

MS was shown to be identical to a protein purified from mouse peritoneal macrophages that bound oxLDL on ligand blots (18). Human monocyte-derived macrophages similarly express an oxLDL binding protein with a strong identity to CD68, the human homolog of MS (45). CD68 expressed in THP-1 cells, a human monocyte-derived cell line, also bound oxLDL in ligand blots (19). We confirmed, as shown by Yoshida and colleagues (26), that both MM-LDL and oxLDL upregulated MS mRNA and protein expression 2- to 3-fold in resident murine peritoneal macrophages. The above data would suggest a role for MS as a macrophage receptor for oxLDL. However, despite strong circumstantial evidence, our studies employing adenoviral-mediated overexpression in cell lines in vitro and mice in vivo, and MS inhibition through gene silencing, do not support a direct role for MS as an oxLDL receptor.

MS is predominantly present in late endosomes and lysosomes in virtually all tissue macrophages (16). Less than 5% of MS is expressed on the resting macrophage cell surface (16), arguing against an important role as a cell surface receptor. Because MS is highly expressed, this small percentage of surface expression may nevertheless be of functional significance. In addition, studies done in thioglycollate-elicited peritoneal macrophages show that 10–15% of MS is expressed on the cell surface and that rapid exchange occurs with intracellular pools (17). These results would not be incompatible with a surface function such as internalization of bound oxLDL. Using adenoviral-mediated expression, we achieved significant levels of surface expression of MS in COS cells, ranging from 37–51% of total expression. This allowed meaningful functional analysis of MS as an oxLDL receptor.

MS possesses unique mucin-like extracellular domains located at the N-terminal region that account for about two thirds of the mass of the mature protein (37). In response to inflammatory stimuli, these regions undergo complex alterations in their patterns of *N*- and *O*-linked glycosylation (15, 38), resulting in size differences evident on SDS-PAGE analysis. The glycosylated regions of MS have been suggested to play a role in protection from the harsh hydrolytic environment found in lysosomes and/or may act as ligands for cell adhesion molecules, such as selectins. We noted that adenoviral-mediated MS expressed in COS-7 cells was ~10 kDa smaller in size compared with the endogenous protein in RAW264.7 cells. We ascribed this to cell-specific differences in glycosylation, and it is interesting to note that there was no size difference when MS was overexpressed in the murine macrophage-like cell lines RAW264.7 and J774.A1. Despite this presumed difference in glycosylation, solubilized MS from AdMS-treated COS-7 and stably transfected CHO cells (the latter data not shown) exhibited the ability to bind oxLDL on ligand blots. These findings agree with previous studies, which showed deglycosylation by treatment with *N*-glycosidase or *O*-glycosidase did not affect the ability of MS or CD68 to bind oxLDL on ligand blots (20, 45). This suggests the binding of oxLDL is not mediated through the carbohydrate moiety.

We compared MS and mCD36 directly as oxLDL receptors using the same adenoviral vector expression system in transfected cells. COS-7 cells expressing mCD36 bound oxLDL with high affinity, indicating that mCD36 is a physiological receptor for oxLDL (8, 27). MS, in contrast, in binding studies at 4°C and 37°C (up to 16 h), showed no evidence of a specific role in oxLDL binding, uptake, and degradation. SR-BI and CD36 are the members of the class B scavenger receptor family. SR-BI also binds oxLDL, but has been better characterized as a receptor for HDL. Both SR-BI and CD36 (less efficiently) mediate the selective uptake of HDL-CE by a process in which HDL delivers CE to the cell without the internalization and lysosomal degradation of the whole HDL particle (46–48). Recent evidence also suggests that hepatic SR-BI selectively takes up oxidized CE from HDL (49). We used CHO cells stably transfected with MS to examine its possible role in the selective uptake of lipids from ^{125}I and ^{3}H double-labeled oxLDL, but failed to find any MS-specific effect.

Because of its selective expression and late endosomal localization in macrophages, MS has been postulated to be important in phagocytosis, and cell-cell and cell-pathogen interactions. Alternatively, its predominantly intracellular location may indicate an important role following receptor-mediated uptake of modified lipoproteins. Definitive studies, however, require the generation of mice deficient in MS. In summary, our data obtained from in vitro and in vivo experiments involving atherogenic diet-induced upregulation and adenoviral-mediated overexpression of MS, as well as inhibition through gene silencing, indicate that this macrophage-specific molecule, despite results from ligand blot studies, does not play a significant direct role in oxLDL metabolism.

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