Effect of Human Scavenger Receptor Class A Overexpression in Bone Marrow–Derived Cells on Cholesterol Levels and Atherosclerosis in ApoE-Deficient Mice

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Abstract—In the arterial wall, scavenger receptor class A (SRA) is implicated in pathological lipid deposition. In contrast, in the liver, SRA is suggested to remove modified lipoproteins from the circulation, thereby protecting the body from their pathological action. The role of SRA on bone marrow–derived cells in lipid metabolism and atherogenesis was assessed in vivo by transplantation of bone marrow cells overexpressing human SRA (MSR1) to apoE-deficient mice. In vitro studies with peritoneal macrophages from the transplanted mice showed that macrophage scavenger receptor function, as measured by cell association and degradation studies with acetylated LDL, was ≈3-fold increased on overexpression of MSR1 in bone marrow–derived cells as compared with control mice. Despite the increased macrophage scavenger receptor function in vitro, no significant effect of MSR1 overexpression in bone marrow–derived cells on the in vivo atherosclerotic lesion development was found. In addition to arterial wall macrophages, liver sinusoidal Kupffer cells also overexpress MSR1 after bone marrow transplantation, which may scavenge atherogenic particles more efficiently from the blood compartment. Introduction of bone marrow cells overexpressing human MSR1 in apoE-deficient mice induced a significant reduction in serum cholesterol levels of ≈20% (P<0.001, 2-way ANOVA) as the result of a decrease in VLDL cholesterol. It is suggested that the reduction in VLDL cholesterol levels is due to increased clearance of modified lipoproteins by the overexpressed MSR1 in Kupffer cells of the liver, thereby protecting the arterial wall against the proatherogenic action of modified lipoproteins. (Arterioscler Thromb Vasc Biol. 2000;20:2600-2606.)

Key Words: scavenger receptor ■ atherosclerosis ■ hyperlipidemia ■ macrophages ■ bone marrow transplantation

An early stage in the development of atherosclerosis is the formation of foam cells that arise from macrophages and smooth muscle cells caused by the excessive uptake of cholesteryl ester–rich lipoproteins.1,2 Several lipoprotein receptors have been characterized that might be involved in the uptake of lipoproteins by these cell types, including the LDL receptor, the VLDL receptor, LDL receptor–related protein (LRP), and several types of scavenger receptors.3,4 Scavenger receptor class A (SRA) was the first scavenger receptor that was identified and the molecular cloning of this receptor disclosed 2 isoforms, type I and type II, which are derived from alternative splicing of a single gene product.5–8 SRA is a trimeric glycoprotein that consists of 6 different domains, including the isoform-specific C-terminal domain and the collagen-like domain, which is involved in ligand binding.9 SRA mediates the uptake and degradation of a broad range of negatively charged ligands and modified lipoproteins, including oxidized and acetylated LDL (OxLDL and AcLDL).10,11 In contrast to the LDL receptor, SRA is not downregulated by intracellular cholesteryl ester accumulation and might therefore play an important role in foam cell formation.3 The expression of SRA in the vessel wall is highly induced during cholesterol feeding and the induction of atherosclerotic lesion formation.3 Immunohistochemical studies in humans, rabbits, and mice demonstrated SRA in atherosclerotic lesions, where it is primarily expressed by macrophages.12,13 However, some studies also suggest that smooth muscle cells do express SRA.14–16 Recently, SRA-deficient mice have been generated by targeted disruption of the SRA gene.17 On an apoE-deficient background, it was found that SRA deficiency results in a reduction of ≈60% of spontaneously developed atherosclerosis, providing evidence that SRA is of prime importance in the development of atherosclerosis, at least in apoE-deficient mice.
In addition to arterial wall macrophages, liver sinusoidal Kupffer cells and endothelial cells also express SRA. Expression of SRA in the liver is thought to form a major protection system of the body by scavenging atherogenic particles from the blood compartment, thereby reducing the accumulation of modified lipoproteins in the interstitial space of the vessel wall. In apoE−/− mice, SRA deficiency increased serum cholesterol levels by 46%. Apparently this increase in serum cholesterol levels did not facilitate atherosclerotic lesion development, possibly as the result of the absence of SRA in macrophages in the arterial wall.

In the present work, we wanted to establish whether overexpression of SRA exclusively in macrophages will influence serum cholesterol levels and the susceptibility to atherosclerosis in apoE-deficient mice. To investigate the effect of macrophage SRA overexpression on atherogenesis, we generated apoE-deficient mice overexpressing human SRA (MSR1) solely in bone marrow–derived cells by transplantation of bone marrow–overexpressing MSR1 to apoE-deficient mice. Macrophages isolated from these transplanted animals show an increased SRA activity as compared with macrophages from control transplanted mice. Surprisingly, despite this increased uptake of modified lipoproteins by macrophages in vitro, no significant effect of MSR1 overexpression in bone marrow–derived cells on in vivo atherosclerosis in apoE−/− mice, SRA deficiency was fasted overnight and blood was subsequently collected under anesthesia by puncture of the orbital plexus. After density gradient ultracentrifugation according to Redgrave et al., the top fraction (d < 1.006 g/mL), containing apoE-deficient β-VLDL, was isolated and dialyzed against PBS/1 mmol/L EDTA. Protein content was determined according to Lowry et al., with bovine serum albumin (BSA) used as an internal standard.

For isolation of apoE-deficient β-VLDL, apoE-deficient mice were oxidatively modified by incubation of 0.2 mg/mL protein with 11 μmol/L CuSO4 for 20 hours at 4°C. Radiolabeling of the lipoproteins with 125I at pH 10.0 was performed according to McFarlane, modified as described earlier.

In Vitro Studies With Peritoneal Macrophages

Five days after intraperitoneal injection of 3% Brewer’s thiglycolate-elicited macrophages were harvested by lavage of the peritoneal cavity with 10 mL sterilized PBS. The isolated macrophages were washed 3 times with sterilized PBS and plated in 24-well plates at a density of 0.5 × 106 cells/500 μL in DMEM, supplemented with 10% (wt/vol) bovine calf serum, 2 mmol/L NaCl, 100 μg/mL streptomycin, and 100 IU/mL penicillin. After 4 hours, nonadhering cells were removed by washing. At 2 days after isolation, cells were incubated with the indicated concentrations of 125I-acetylated (Ac)LDL, 125I-oxidized (Ox)LDL, or 125I-Ox-β-VLDL. After 3 hours at 37°C, cells were washed and lysed in 0.1 mol/L NaOH, and the cell protein content was determined according to Lowry et al. to determine the cell association per milligram of cell protein. Degradation products in the medium were measured by addition of 0.4 mL 35% trichloroacetic acid to 0.5 mL medium. After incubation at 4°C for 30 minutes, 0.25 mL of 0.7 mol/L AgNO3 was added, samples were centrifuged for 5 minutes at 16 000g, and the radioactivity was determined in the supernatant.

To study the effect of MSR1 overexpression in oxidized β-VLDL–induced foam cell formation, macrophages from MSR1-transgenic and wild-type mice were isolated as described above. At 2 days after the isolation, the cells were incubated with the indicated amounts of oxidized β-VLDL in DMEM/2% BSA for 24 hours at 37°C. After 24 hours, cells were washed and fixed with 2.2% PBS-buffered formaldehyde, and accumulated lipids were stained with 0.5% oil red O in propylene glycol. Oil red O staining was quantified with a light microscope connected to a full-color video camera and running Leica Qwin Imaging Software.

Histological Analysis of Hearts and Aortas for Atherosclerosis

To analyze the development of atherosclerosis, transplanted mice were killed at 12 weeks after BMT. Hearts and aortas were perfused in situ with oxygenated Krebs buffer (37°C, 100 mm Hg) for 20 to 30 minutes through a cannula in the left ventricle, followed by perfusion with 3.7% neutral-buffered formalin (Formal-fix, Shandon Scientific Ltd) for 30 minutes. Hearts and aortas were excised and stored in formalin.
To evaluate the development of atherosclerotic lesions, the aortas were separated from the hearts. Hearts were bisected at the level of the atria, and the base of the heart plus aortic root were taken for analysis. Cryostat 10-μm cross sections of the aortic root were made and stained with oil red O (BDH Ltd). The atherosclerotic lesion area in the sections was quantified with a light microscope connected to a full-color video camera and Optimas 6.1 image analysis software (BioScan). The mean atherosclerotic lesion area for each individual mouse was calculated (in μm²) from 10 sections, starting at the appearance of the tricuspid valves, as described previously.31,32

The perfusion-fixed aortas were used to analyze atherosclerosis development over the whole length of the aorta. After dissection of the adventitial fat, the aorta was stained for vascular lipids with oil red O. The presence of oil red O–positive lesions was subsequently studied with the use of a stereomicroscope.

Serum Cholesterol and Triglyceride Analysis
After an overnight fasting period (15 hours), ~100 μL blood was drawn from each individual mouse by tail bleeding. The concentrations of total cholesterol and triglycerides in serum were determined by enzymatic procedures (Boehringer Mannheim). Precipath (standardized serum; Boehringer Mannheim) was used as an internal standard. The distribution of cholesterol over the different lipoproteins in serum was determined by loading of 30 μL serum from each mouse onto a Superose 6 column (3.2×30 mm, Smart-system, Pharmacia). Serum was fractionated at a constant flow rate of 50 μL/min, with the use of PBS. Total cholesterol content of the effluent was determined enzymatically.

Statistical Analysis
Statistically significant differences in repeated measurements were tested by means of ANOVA in time, and individual comparisons were made by means of the unpaired Student’s t test (Instat Graphpad software). A value of *P<0.05 was regarded as significant.

Results
To gain insight into the in vivo effect of MSR1 overexpression in bone marrow–derived cells on atherosclerotic lesion development, female recipient apoE-deficient mice were transplanted with apoE-deficient bone marrow overexpressing MSR1 after lethal irradiation.

Detection of MSR1 Expression After BMT
Repopulation of bone marrow and liver with donor-derived cells was determined by polymerase chain reaction analysis of the expression of MSR1 in bone marrow and liver at 12 weeks after BMT. As expected, no MSR1 DNA was detected in mice that received syngeneic bone marrow from apoE−/− mice. In mice transplanted with apoE−/− MSR1 bone marrow, an MSR1-specific band of 350 bp appeared in DNA from bone marrow (data not shown), indicating that MSR1 bone marrow cells had successfully repopulated the recipient mice. Furthermore, MSR1 expression was also demonstrated in the liver, indicating that the Kupffer cells of the liver also were replaced (data not shown).

Effect of Human Macrophage Scavenger Receptor Class A Overexpression in Bone Marrow–Derived Cells on Modified Lipoprotein Cell Association and Degradation
To demonstrate that the successful repopulation of the transplanted mice with MSR1 bone marrow resulted in a functionally overexpressed MSR1, thioglycollate-elicted macrophages were isolated from both transplantation groups at 12 weeks after BMT. Subsequently, the cell association and degradation of acetylated LDL, a high-affinity ligand for the scavenger receptor, by these macrophages was determined. The cell association and degradation of AcLDL was highly increased in macrophages from apoE-deficient mice transplanted with apoE−/− MSR1 bone marrow as compared with macrophages from control transplanted mice, indicating that macrophages indeed functionally overexpress MSR1 and therefore were replaced successfully. The maximal cell association was 2.7-fold increased from 828±170 ng/mg in macrophages (mϕ) from apoE−/−→apoE−/− mice to 2261±348 ng/mg in mϕ from apoE−/− MSR1→apoE−/− animals (Figure 1A). The maximal degradation capacity was 4-fold increased (Figure 1B). For comparison, the association and degradation of AcLDL by mϕ, isolated from the donor strains of mice, was 1.5-fold and 4.4-fold increased in mϕ overexpressing MSR1 as compared with control mice (data not shown). The comparable association and degradation rates of AcLDL by macrophages isolated from the transplanted mice and from the donor strains of mice thus indicate that the replacement of tissue macrophages by cells from donor origin is complete. This is in accordance with previously published data by our group showing that after BMT, the degree of chimerism is complete in bone marrow and spleen.25

In the apoE-deficient mice that were used as recipients for our BMT studies, β-VLDL is the most predominant lipoprotein. Oxidatively modified β-VLDL in the subendothelial

Figure 1. Effect of human macrophage SRA overexpression in bone marrow–derived cells on cell association and degradation of AcLDL. Thioglycollate–elicited peritoneal macrophages were incubated with increasing concentrations of 125I-AcLDL at 37°C for 3 hours. Cell association (A) and degradation (B) were determined in macrophages from apoE-deficient mice transplanted with apoE−/− (○) or apoE−/− MSR1 (●) bone marrow. Values are mean±SD of macrophages of 3 individual mice. *Significant difference (*P<0.01) vs macrophages from control transplanted mice.
space of the arterial wall will presumably be involved in atherosclerotic lesion formation in these mice. Therefore, the effect of MSR1 overexpression in macrophages on the association and degradation of oxidatively modified apoE-deficient \( \beta \)-VLDL was established. To discriminate between any possible species differences in lipoprotein recognition by the human scavenger receptor, the association and degradation of oxidatively modified apoE-deficient murine \( \beta \)-VLDL was also compared with oxidatively modified human LDL (Figure 2). Oxidative modification of apoE-deficient \( \beta \)-VLDL induced a 3.5-fold increase in the association to macrophages as compared with native apoE-deficient \( \beta \)-VLDL (data not shown). Overexpression of MSR1 in macrophages induced a 33% and 32% increase in the association and degradation of oxidized apoE-deficient murine \( \beta \)-VLDL, respectively. A similar effect was observed on the association (29% increase) and degradation (21% increase) of oxidized human LDL, indicating that the overexpressed human MSR1 recognizes human as well as murine modified lipoproteins.

The effect of MSR1 overexpression in macrophages on the uptake of OxLDL is less pronounced as compared with the effect on AcLDL, which is probably caused by the presence of additional receptors for OxLDL besides scavenger receptor class A.\(^3\) To demonstrate that the increased metabolism of oxidized apoE-deficient \( \beta \)-VLDL by macrophages from MSR1 transgenic mice results in increased cholesterol accumulation, macrophages were analyzed in vitro for foam cell formation by staining of the accumulated lipids with oil red O. Determination of the oil red O staining per cell area revealed that lipid accumulation in macrophages from MSR1-transgenic mice was 1.6-fold increased as compared with macrophages from wild-type mice, indicating that macrophages overexpressing human MSR1 are more prone to convert into foam cells on incubation with murine lipoproteins (Figure 3).

**Effect of Human Macrophage Scavenger Receptor Class A Overexpression in Bone Marrow–Derived Cells on Susceptibility to Atherosclerosis**

To gain insight into the effect of human macrophage scavenger receptor class A overexpression in bone marrow–derived cells on susceptibility to atherosclerosis, we analyzed the atherosclerotic lesion area in the aortic root of the transplanted mice at 12 weeks after BMT. Representative photomicrographs of lipid-rich atherosclerotic lesions in the aortic root are shown in Figure 4. Despite the increased association of modified lipoproteins in vitro, no significant difference in the mean atherosclerotic lesion area could be demonstrated between apoE\(^{-/-}\) MSR1→apoE\(^{-/-}\) mice (2.94\(\pm\)0.85\(\times\)10\(^5\) \(\mu\)m\(^2\); \(n=6\)) as compared with apoE\(^{-/-}\)→apoE\(^{-/-}\) animals (3.83\(\pm\)0.69\(\times\)10\(^5\) \(\mu\)m\(^2\); \(n=7\)) (Figure 5).
Figure 5. Effect of human macrophage SRA overexpression in bone marrow–derived cells on susceptibility to atherosclerosis. Mean atherosclerotic lesion area was calculated from oil red O–stained cross sections of aortic root at level of tricuspid valves. Values indicate mean lesion area of 10 sections in each mouse. No significant difference was observed between 2 groups.

In addition to quantitative analysis of atherosclerosis in multiple sections in the aortic root, atherosclerosis development was also studied qualitatively over the total length of the aorta (data not shown). The anatomic distribution of atherosclerotic lesions was identical in both transplantation groups. Lesions were present in the inner curvature of the aortic arch and at branch points with the carotid arteries in both groups of mice. Furthermore, along the descending aorta, atherosclerotic lesions were mainly observed near the orifice of the smaller branching arteries.

Effect of Human Macrophage Scavenger Receptor Class A Overexpression in Bone Marrow–Derived Cells on Serum Lipid Levels

In addition to arterial wall macrophages, Kupffer cells overexpress MSR1 after BMT. Because expression of SRA in the liver is suggested to form a major protection system of the body by scavenging atherogenic particles from the blood compartment, the effect of MSR1 overexpression in bone marrow–derived cells on serum lipid levels was determined. The effect of transplantation of apoE-deficient mice with bone marrow from apoE-deficient mice (apoE$^{−/−}$apoE$^{−/−}$) and apoE-deficient mice overexpressing the human SRA (apoE$^{−/−}$MSR1$^{+/+}$apoE$^{−/−}$) on serum cholesterol levels is depicted in Figure 6. Even on a normal chow diet, apoE-deficient mice demonstrate a marked hyperlipidemia. Introduction of bone marrow–derived cells overexpressing MSR1 in apoE-deficient mice induced a significant reduction in serum cholesterol concentrations of $\approx 20\%$ ($P<0.001$, 2-way ANOVA) in the weeks after BMT. No significant effect of BMT on serum triglyceride levels was found (data not shown). Analysis of the distribution of cholesterol over the different lipoprotein fractions at 4 weeks after BMT revealed that the reduction in serum cholesterol levels caused by overexpression of MSR1 on bone marrow–derived cells in apoE-deficient mice was caused by a significant ($P<0.05$) reduction in VLDL cholesterol (Figure 7).

Discussion

SRA is implicated in the pathological lipid deposition and subsequent transformation of macrophages to foam cells.$^{1,2}$ Recently, it has been demonstrated that targeted disruption of the SRA gene in apoE-deficient mice results in a 60% decrease in the mean atherosclerotic lesion area, indicating that SRA is of prime importance for the development of atherosclerosis in these mice.$^{17}$ Several lipoprotein receptors have been characterized that might be involved in the formation of foam cells, including the LDL receptor, the VLDL receptor, LRP, and several types of scavenger receptors.$^{3,4}$ However, when apoE, a high-affinity ligand for the LDL receptor, the VLDL receptor, and LRP is absent, the relative contribution of the scavenger receptors to foam cell formation is expected to be most evident. The data of the present study demonstrate that atherosclerotic lesion formation in apoE-deficient mice is not significantly affected by human scavenger receptor class A overexpression in bone marrow–derived cells, despite an increased cell association and degradation of modified lipoproteins in vitro by macrophages. These data therefore indicate that the increased capacity of the SRA to metabolize modified lipoproteins does not result in the anticipated increase in atherosclerotic lesion formation. In contrast to the decreased susceptibility of apoE-deficient mice on targeted disruption of the SRA gene, De Winther et al.$^{34}$ recently demonstrated that SRA deficiency on an apoE3-Leiden background results in even more severe lesions.$^{17,34}$ In addition, we now demonstrate that overexpression of MSR1 in bone marrow–derived cells does not induce...
an increase in atherosclerotic lesion development, indicating that the role of SRA in atherogenesis is complex.

After BMT, not only arterial wall macrophages but also liver sinusoidal Kupffer cells are replaced by cells of donor origin. Because expression of SRA in the liver is thought to form a major protection system of the body by scavenging atherogenic particles from the blood compartment, the effect of introduction of bone marrow–derived cells overexpressing MSR1 into apoE-deficient mice on serum lipid levels was determined. Overexpression of MSR1 in bone marrow–derived cells induced a significant reduction in serum cholesterol levels. The observed reduction in serum cholesterol in apoE/−/− MSR1→apoE/−/− mice was confined to the VLDDL-sized fraction, implicating an involvement of SRA in the clearance of VLDDL-sized lipoproteins. Suzuki et al17 demonstrated that disruption of the SRA gene in apoE-deficient mice results in significantly higher serum cholesterol levels.

In apoE-deficient mice, it was shown that plasma lipid peroxidation is enhanced because of increased VLDDL and LDL lipid peroxidation.35,36 Furthermore, high titers of autoantibodies recognizing epitopes of oxidized lipoproteins were demonstrated in these mice.37 Therefore, it is most likely that the decrease in VLDDL cholesterol levels in apoE/−/− MSR1→apoE/−/− mice as compared with apoE/−/− animals is due to uptake of oxidized VLDDL by the overexpressed MSR1 on macrophages. Especially macrophages of the liver, Kupffer cells, may be involved in this process because these cells quantitatively form the major removal site for modified lipoproteins. Wölle et al39 showed that overexpression of bovine SRA type I in the mouse liver hepatocytes suppressed the diet-induced high levels of apoB-containing lipoproteins and led to an enhanced biliary secretion of cholesterol and bile acids. Together with the data of Suzuki et al17 and our present data, it is evident that scavenger receptor expression in the liver influences serum cholesterol levels, at least in apoE-deficient mice.

In conclusion, MSR1 overexpression in bone marrow–derived cells in apoE-deficient mice does not affect atherosclerotic lesion development, whereas VLDDL cholesterol levels were significantly reduced. We suggest that the overexpression of MSR1 in bone marrow–derived cells reduces serum VLDDL cholesterol levels by increasing the clearance of oxidatively modified lipoproteins through the overexpressed MSR1 in Kupffer cells of the liver, thereby protecting the arterial wall against the proatherogenic action of these oxidized lipoproteins.

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