Effect of human scavenger receptor class A overexpression in bone marrow-derived cells on lipoprotein metabolism and atherosclerosis in low density lipoprotein receptor knockout mice

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Abstract Scavenger receptors, which include various classes, play an important role in atherogenesis by mediating the unrestricted uptake of modified lipoproteins, resulting in the massive accumulation of cholesteryl esters. Because macrophage-derived foam cells are considered to be an important feature in early atherogenesis, we investigated the role of scavenger receptor class A (SR-A) overexpression, especially on macrophages in lipoprotein metabolism and atherosclerosis. Bone marrow from human SR-A (MSR1)-overexpressing mice was transplanted into irradiated low density lipoprotein receptor knockout [LDLR(-/-)] mice. The transplantation resulted in an increase in total serum cholesterol (approximately 15 to 25%), especially in the VLDL fraction, when compared with LDLR(-/-) mice that were transplanted with bone marrow of wild-type littermates. Quantification of atherosclerotic lesions in the mice that were fed a “Western-type” diet for 3 months revealed that there were no differences in mean lesion area between LDLR(-/-) mice transplanted with MSR1 overexpressing and wild-type littermate bone marrow, despite increased scavenger receptor activity in vitro. The presence or absence of the LDLR in the transplanted bone marrow did not influence these results. In conclusion, introduction of MSR1-overexpressing bone marrow in LDLR(-/-) mice via bone marrow transplantation resulted in a slight increase in lipoprotein levels, but had no effect on the atherosclerotic lesion area, despite increased scavenger receptor activity in vitro. —Herijgers, N., Menno P. J. de Winther, Miranda Van Eck, Louis M. Havekes, Marten H. Hofker, Peter M. Hoogerbrugge, and Theo J. C. Van Berkel.

Supplementary key words LDL receptor • scavenger receptor class A • atherosclerosis • macrophages • bone marrow transplantation

Macrophage-derived foam cells play an important role in the initiation and progression of atherosclerosis (1). These lipid-laden macrophages are thought to be the result of unrestricted uptake of oxidatively modified lipoproteins, such as oxidized low density lipoproteins (LDL), through scavenger receptors (2, 3). The existence of scavenger receptors was originally demonstrated by Goldstein et al. (4) during their attempts to learn how cholesterol from low density lipoproteins accumulates in atherosclerotic plaques of patients with familial hypercholesterolemia, who lack a functional LDL receptor (LDLR). Since then several classes of scavenger receptors have been identified and the scavenger receptor family is still growing (5, 6). The first scavenger receptor to be cloned and characterized was the scavenger receptor class A (SR-A). It is a trimeric membrane glycoprotein consisting of six structural domains including the collagen-like domain, which is involved in ligand binding (7–9). Three isoforms, types I, II, and III, are produced from a single gene by alternative splicing (10–12). The expression of SR-A has been detected in macrophages in vitro (7, 13) and in vivo (14–16). Also, endothelial cells in liver, aorta, and adrenal gland were shown to express SR-A (17, 18). However, Gough et al. (19) demonstrated that in human and rabbit atherosclerotic lesions, SR-A is mainly expressed by macrophage-derived foam cells, whereas only a low level of expression was found in the

Abbreviations: apo, apolipoprotein; BSA, bovine serum albumin; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LDLR, LDL receptor; SRA, scavenger receptor class A; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; VLDL, very low density lipoprotein.

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aortic endothelium. Inconsistent data also exist about the expression of SR-A in smooth muscle cells: Some groups were able to detect SR-A in this cell type (19–21) whereas others did not find any expression (16, 22).

The importance of SR-A in atherosclerosis has been demonstrated in mice that are deficient in SR-A (23). On an apolipoprotein E (apoE)-deficient background, these mice showed a 60% reduction in atherosclerosis despite an increase in plasma cholesterol levels. Also in the absence of the LDLR, SRA deficiency resulted in a 20% decrease in atherosclerotic lesion area (24).

In the present study we investigated the role of SR-A in atherosclerosis and lipoprotein metabolism, when it is exclusively expressed on macrophages. We therefore transplanted bone marrow of human SR-A type I and II-overexpressing (MSR1) mice into LDLR-deficient mice. Our results indicate that macrophage-specific overexpression of SR-A does influence lipid metabolism, but not atherosclerotic lesion formation.

**MATERIALS AND METHODS**

**Mice**

Human scavenger receptor class A type I and II (MSR1) transgenic mice were generated by oocyte microinjection of a 180-kb yeast artificial chromosome containing the MSR1 with 60- and 40-kb flanking sequences at the 5’ and 3’ end, respectively, thereby including its natural promotor and all other native regulatory elements, as described previously (25). Transgenic mice were identified by polymerase chain reaction (PCR) analysis of genomic tail DNA. Because all natural regulatory elements are included, the MSR1 gene is expressed according to the natural pattern in these mice, with the highest expression in macrophages and organs containing high amounts of macrophages, such as liver and lungs. These mice were hybrids between BCBA, 129Sv, and C57BL/6 strains (N3 generation of backcrosses to C57BL/6).

Homozygous LDL receptor knockout mice [LDLR(−/−)] were obtained from the Jackson Laboratory (Bar Harbor, ME) as mating pairs, and bred in the Gaubius Laboratory (Leiden, The Netherlands). These mice were hybrids between the C57BL/6 and 129Sv strains (N4 generation of backcrosses to C57BL/6). C57BL/6 mice were obtained from the Broekman Institute (Somenner, The Netherlands). MSR1 mice were cross-bred with LDLR(−/−) mice to obtain MSR1.LDLR(−/−) mice.

The animals were housed in sterile filter-top cages and were fed standard rat/mouse chow (SRM-A; Hope Farms, Woerden, The Netherlands). For the analysis of atherosclerosis, the mice were fed a sucrose-based semisynthetic “Western-type” diet described (27). Atherosclerotic lesions in the sections were quantified with a light microscope connected to a video camera and running Leica (Cambridge, UK) QWin Imaging software. Mean lesion area was calculated from the first 10 sections in the direction of the aortic arch from the point where all three aortic valve leaflets first appear, as previously described (27).

**Isolation of human LDLR, acetylation, and incubation with murine peritoneal macrophages**

Human LDLR was isolated from healthy volunteers as described by Redgrave et al. (28). After density ultracentrifugation, LDL (1.019 g/mL < d < 1.063 g/mL) was collected and dialyzed against PBS–1 mm EDTA. Protein content was determined according to Lowry et al. (29) with bovine serum albumin (BSA) as an internal standard. LDL was acetylated according to Basu et al. (30) and subsequently labeled with [125I] at pH 10.0 according to McFarlane (31), modified as described earlier (32). Five days after intraperitoneal injection of 3% Brewer’s thioglycollate medium, macrophages were harvested by lavage of the peritoneal cavity with 10 mL of PBS. The isolated macrophages were washed three times with sterilized PBS and plated in 24-well plates at a density of 0.5 × 10⁶ cells/500 μL in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with line (PBS). The cells were washed twice with PBS. Recipients received 1 × 10⁷ bone marrow cells by tail vein injection 24 h after irradiation.

**PCR analysis**

Bone marrow and liver tissue was isolated 12 weeks after bone marrow transplantation. DNA, isolated from these tissues, was subsequently tested by PCR for the presence of the transgene, using the following MSR1-specific primers:

Forward: 5′-GAAGATGTGGAGTCCATTGC3′
Reverse: 5′-TGGAGCCAATACTGGATGC3′

**Serum cholesterol and triglyceride analysis**

After an overnight fasting period, small blood samples (50 μL) were obtained by tail bleeding. The concentrations of total cholesterol and triglycerides in the sera were determined by enzymatic procedures (Boehringer Mannheim Biochemicals, Mannheim, Germany). Precipath (standardized serum) was used as an internal standard (Boehringer Mannheim Biochemicals).

The distribution of cholesterol over the different lipoproteins was determined by loading 30 μL of serum from each mouse onto a Superose 6 column (3.2 × 30 mm, Smart-system; Pharmacia, Uppsala, Sweden). The serum was fractionated at a constant flow rate of 50 μL/min, using phosphate-buffered saline containing 1 mm EDTA. Twenty-eight fractions of 50 μL were collected. The fractions were assayed for their total cholesterol content.

**Analysis of atherosclerosis**

The mice were killed and the heart and vascular tree were perfused in situ with oxygenated Krebs Ringer bicarbonate buffer at 37°C under a pressure of 100 mmHg for 30 min via a cannula in the left ventricle. The buffer was then replaced by 3.7% neutral-buffered formalin (3.7% formaldehyde, Formal-Fixx; Shandon Scientific, Runcorn, Cheshire, UK) and the tissue was fixed during a perfusion of 30 min. Hearts and aortas were excised and stored in formalin. The hearts were bisected just below the atria and the base of the heart and aortic root were taken for analysis. Cryostat 10-μm cross-sections of the aortic root were then taken and stained with oil red O as described previously (27). Atherosclerotic lesions in the sections were quantified with a light microscope connected to a video camera and running Leica (Cambridge, UK) QWin Imaging software. Mean lesion area was calculated from the first 10 sections in the direction of the aortic arch from the point where all three aortic valve leaflets first appear, as previously described (27).
10% (w/v) bovine calf serum, l-glutamine (2 mmol/L), streptomycin (100 μg/mL), and penicillin (100 IU/mL). After 4 h, nonadhering cells were removed by washing. Two days after isolation, cells were incubated with increasing concentrations of 125I-labeled acetylated LDL (AcLDL). After 3 h at 37°C cells were washed and lysed in NaOH (0.1 mol/L) and the cell protein content was determined according to Lowry et al. (29) to determine the cell association of 125I-labeled AcLDL per mg of cell protein. Degradation products of 125I-labeled AcLDL in the medium were measured by addition of 0.4 mL of 35% trichloroacetic acid to 0.5 mL of medium. After incubation at 4°C for 30 min, 0.25 mL of AgNO₃ (0.7 mol/L) was added, samples were centrifuged for 5 min at 16,000 g, and the radioactivity was determined in the supernatant.

Statistical analysis

Statistical analysis of the data was performed by two-way analysis of variance (ANOVA), with the Student-Newman-Keuls multiple comparison test as post-test when P < 0.05.

RESULTS

In the present study we investigated the effect of SR-A overexpression in bone marrow-derived cells on lipoprotein metabolism and atherosclerosis. Therefore, we transplanted bone marrow of human SR-A (MSR1)-overexpressing mice in the presence or absence of the LDLR into irradiated LDLR(-/-) mice. Control transplantations with bone marrow of normal SR-A-expressing littermates were also included. Previously, we showed that the method of bone marrow transplantation in mice results in an almost complete replacement of recipient hemopoietic cells by those of donor origin (33).

Detection of MSR1 expression after BMT

Repopulation of the recipients with donor-derived cells was determined by PCR analysis of the expression of MSR1 in bone marrow 12 weeks after bone marrow transplantation (BMT). No MSR1 DNA could be detected in mice that received bone marrow from wild-type (wt) mice, whereas an MSR1-specific band of 350 bp appeared in mice transplanted with wt.MSR1 bone marrow, indicating that MSR1 bone marrow cells had successfully repopulated the recipient mice (data not shown).

Effect of BMT on serum lipoprotein levels

During the weeks after BMT, total serum cholesterol and triglyceride levels of the transplanted mice were repeatedly determined. The results are shown in Figs. 1 and 2.

The total serum cholesterol levels at various time points in MSR1. wt→LDLR(-/-) transplanted mice were significantly higher than in the wt→LDLR(-/-) transplanted mice (P < 0.0001) (Fig. 1A). When the LDLR was absent in the bone marrow, similar results were obtained (P < 0.0001) (Fig. 1B). At 8 weeks after BMT the mice were given a semisynthetic Western-type diet for 3 months. The diet caused an average increase in serum cholesterol levels of approximately fivefold in all groups of mice.

At week 4 and 6 after BMT the triglyceride levels in all groups of transplanted mice decreased temporarily (Fig. 2). Because this decrease is present in both experimental and control groups, it probably reflects an effect of the BMT procedure. During the experiment, MSR1. wt→LDLR(-/-)
transplanted mice had higher triglyceride levels than wt→LDLR(−/−) transplanted mice (P < 0.001) (Fig. 2A). The mice that were transplanted with LDLR(−/−) bone marrow, however, did not reveal any significant differences between the groups that were transplanted with MSR1 bone marrow or control bone marrow (Fig. 2B). No effect of the diet on serum triglyceride levels in the mice was observed.

Effect of BMT on distribution of serum lipoprotein cholesterol

The effect of BMT on the distribution of serum cholesterol over the different lipoprotein fractions was analyzed by liquid chromatography. At 8 weeks after BMT, no significant difference between the four groups of transplanted mice could be observed (Fig. 3A and B). The plasma contained a considerable amount of cholesterol in the very low density lipoprotein (VLDL) and intermediate/low density lipoprotein (IDL/LDL) fraction, next to the high density lipoprotein (HDL) fraction, as is characteristic of LDLR(−/−) mice.

At 14 weeks after BMT (6 weeks on a Western-type diet), the transplanted mice showed a large increase in especially VLDL cholesterol (Fig. 3C and D). The MSR1.wt→LDLR(−/−) transplanted mice had a larger VLDL peak than did the control wt→LDLR(−/−) mice (P < 0.01) (Fig. 3C). Surprisingly, the mice that were transplanted with LDLR(−/−) bone marrow did not reveal any differences between MSR1 overexpressing and control bone marrow (Fig. 3D).

Effect of BMT on atherosclerosis

To investigate the effects of MSR1 overexpression in bone marrow-derived cells on atherosclerotic lesion formation, the transplanted mice were fed a Western-type diet. After 3 months of diet, the hearts and aortas were perfused and fixed. Cross-sections of the aortic root were examined, and representative photomicrographs of the aortic valves are shown in Fig. 4. The mean lesion area in the aortic root was calculated and is presented in Fig. 5. Overexpression of the MSR1 in bone marrow-derived cells did not result in a significant difference in mean lesion area [MSR1.wt→LDLR(−/−): 9.9 (±1.0) × 10^3; wt→LDLR(−/−): 8.4 (±1.7) × 10^3; MSR1.LDLR(−/−)→LDLR(−/−): 9.6 (±1.7) × 10^3; LDLR(−/−)→LDLR(−/−): 10.0 (±1.3) × 10^3 (mean μm² ± SD)]. Also, the presence or absence of the LDLR in the bone marrow did not make any difference for the atherosclerotic lesion area.

Effect of MSR1 overexpression on the metabolism of acetylated LDL by peritoneal macrophages

To investigate whether MSR1 was indeed functionally overexpressed in macrophages, thioglycollate-elicited macrophages were isolated from mice transplanted with wt and wt.MSR1 bone marrow 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 acetylated LDL was highly increased in macrophages from mice transplanted with wt.MSR1 bone marrow as compared with mice transplanted with wt bone marrow. The maximal cell association was 2.9-fold increased from 368 ± 59 ng/mg in macrophages from mice transplanted with wt bone marrow to 1,056 ± 93 ng/mg in macrophages from mice transplanted with wt.MSR1 bone marrow (Fig. 6A). The maximal degradation capacity was 2.7-fold increased from 4,362 ± 518 ng/mg in macrophages from mice transplanted with wt bone mar-
row to 11,870 ± 1,416 ng/mg in macrophages from mice transplanted with wt.MSR1 bone marrow (Fig. 6B).

**DISCUSSION**

Scavenger receptors are implicated in the uptake of modified lipoproteins by macrophages, resulting in the massive accumulation of cholesteryl esters. SR-A was the first scavenger receptor to be cloned and characterized (7, 8). Its role in atherosclerosis was clearly demonstrated by SR-A(−/−) mice that were crossed with apoE(−/−) mice; they showed a 60% reduction in atherosclerotic lesion area when compared with control apoE(−/−) mice (23). Because expression of the SR-A has been demonstrated not only on macrophages, but also on endothelial cells (17, 18) and smooth muscle cells (19, 20), we transplanted bone marrow of MSR1-overexpressing mice to LDLR(−/−) mice to discriminate between the contributions of these different cell types. Bone marrow transplantation provides a unique method to study the role of an individual gene product from macrophages in various biological pro-

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Fig. 3. Effect of BMT on the distribution of serum lipoprotein cholesterol over various lipoprotein fractions in LDLR(−/−) mice. The mice were transplanted with MSR1.wt (solid circles; n = 9), wt (open circles; n = 8), MSR1.LDLR(−/−) (solid squares; n = 9), or LDLR(−/−) (open squares; n = 8) bone marrow. Sera of individual, fasted mice were analyzed on a Pharmacia Smart column 8 weeks after BMT [standard chow diet; (A) and (B)] and at 14 weeks after BMT [6 weeks on a Western-type diet containing 0.25% cholesterol and 15.0% fat; (C) and (D)]. Fractions 3 to 6 represent VLDL and chylomicrons; fractions 7 to 14, IDL and LDL; fractions 15 to 20, HDL. Values are means ± SEM.
cesses. We used this technique successfully to elucidate the role of the LDLR and apoE production by macrophages in lipoprotein metabolism and atherosclerosis (33, 34).

Transplantation of MSR1\(_{wt}\) bone marrow to LDLR\((-/-)\) mice resulted in an increase in total serum cholesterol, especially in the VLDL fraction, when compared with the control transplantation. The presence or absence of the LDLR in the transplanted bone marrow did not influence this significant difference. Sakaguchi et al. (24) found a decrease in the cholesterol levels in SR-A\((-/-)\) mice that were crossed with LDLR\((-/-)\) mice. It appears, thus, that SRA expression positively correlates with cholesterol levels in LDLR\((-/-)\) mice.

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Transplantation of MSR1 bone marrow results in an overexpression of MSR1 in macrophages. By PCR analysis we have demonstrated that after transplantation MSR1 is overexpressed in bone marrow. MSR1 overexpression in bone marrow resulted in an approximately threefold increase in scavenger receptor activity of peritoneal macrophages, as determined by association and degradation studies using thioglycollate-elicited macrophages. In the present study, we investigated the impact of this overexpression on aortic atherosclerotic lesion formation in MSR1 and wild-type transplanted LDLR\((-/-)\) mice that were fed a Western-type diet for 3 months. Despite the observed increase in serum cholesterol and triglyceride levels, no effect of MSR1 overexpression on atherosclerotic lesion development was observed. However, when the mean atherosclerotic lesion area is corrected for the increase in serum cholesterol levels even a slight decrease in the mean atherosclerotic lesion area is observed and not the anticipated increase in susceptibility. Data from De Winther et al. (35) confirm this possible antiatherogenic effect of the SRA: They demonstrated that on an APOE3 Leiden background SR-A deficiency results even in more severe lesions. In contrast, in
SRA\(^{-/-}\), LDLR\(^{-/-}\) mice, Sakaguchi et al. (24) found a 20% reduction of atherosclerotic lesion area when compared with control LDLR\(^{-/-}\) mice. These contradictory results indicate that the role of SR-A in atherogenesis is complex and might depend on additional factors, such as the genetic background of the animal model used. From our data it can be concluded that normal SR-A expression on macrophages in LDLR\(^{-/-}\) mice already provides the maximal capacity needed for the development of atherosclerosis. In the present study, atherosclerotic lesion development was determined after 3 months of feeding a Western-type diet starting at 2 months after BMT. Because of the high susceptibility of LDLR\(^{-/-}\) mice to diet-induced atherosclerosis, the analyzed atherosclerotic lesions were complex. Incubation of peritoneal macrophages from the transplanted mice with acetylated LDL showed that overexpression of MSR1 induces the uptake of acetylated LDL by these cells. Thus, MSR1 overexpression is expected to affect foam cell formation. Therefore, we cannot exclude that MSR1 overexpression does affect the rate of lesion development and that effects of MSR1 overexpression are different when analyzed in fatty streak lesions. Furthermore, it might also be possible that overexpression of MSR1 is compensated by decreased expression of other macrophage scavenger receptors, such as MARCO, CD68/macrosialin, and CD36 (24).

In both lipoprotein metabolism and atherosclerosis the presence or absence of the LDLR in the transplanted bone marrow did not influence the results. This is in agreement with our previous study, in which transplantation of C57BL/6 bone marrow into LDLR\(^{-/-}\) mice resulted in only a temporary decrease in plasma cholesterol and no effect on atherosclerosis (33). The present study adds to this that the LDLR does not modulate the effects of macrophage-specific MSR1 overexpression.

In conclusion, we have shown that MSR1 overexpression in bone marrow-derived cells results in a significant increase in serum cholesterol and triglycerides, but does not influence atherosclerotic lesion area, indicating that the capacity of MSR1 in LDLR\(^{-/-}\) mice is not rate limiting for atherosclerotic lesion formation.
REFERENCES