Inactivation of Macrophage Scavenger Receptor Class B Type I Promotes Atherosclerotic Lesion Development in Apolipoprotein E–Deficient Mice

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Background—Scavenger receptor class B type I (SR-BI) is expressed in macrophages, where it has been proposed to facilitate cholesterol efflux. However, direct evidence that the expression of macrophage SR-BI is protective against atherosclerosis is lacking. In this study, we examined the in vivo role of macrophage SR-BI in atherosclerotic lesion development in the apolipoprotein (apo) E–deficient mouse model.

Methods and Results—ApoE-deficient mice with (n=16) or without (n=15) expression of macrophage SR-BI were created by transplanting lethally irradiated apoE-deficient mice with bone marrow cells collected from SR-BI+/− apoE−/− mice or SR-BI+/+ apoE−/− mice. The recipient mice were fed a chow diet for 12 weeks after transplantation for analysis of atherosclerosis. Quantification of macrophage SR-BI mRNA by real-time reverse transcription–polymerase chain reaction indicated successful engraftment of donor bone marrow and inactivation of macrophage SR-BI in recipient mice reconstituted with SR-BI+/− apoE−/− bone marrow. There were no significant differences in plasma lipid levels, lipoprotein distributions, and HDL subpopulations between the 2 groups. Analysis of the proximal aorta demonstrated an 86% increase in mean atherosclerotic lesion area in SR-BI+/− apoE−/− mice compared with SR-BI+/+ apoE−/− apoE−/− mice (109.50±18.08 versus 58.75±9.58×10^3 μm^2; mean±SEM, P=0.017). No difference in cholesterol efflux from SR-BI+/+ apoE−/− or SR-BI+/− apoE−/− macrophages to HDL or apoA-I discs was detected.

Conclusions—Expression of macrophage SR-BI protects mice against atherosclerotic lesion development in apoE-deficient mice in vivo without influencing plasma lipids, HDL subpopulations, or cholesterol efflux. Thus, macrophage SR-BI plays an antiatherogenic role in vivo, providing a new therapeutic target for the design of strategies to prevent and treat atherosclerosis. (Circulation. 2003;108:2258-2263.)

Key Words: macrophages ■ receptors ■ cholesterol ■ atherosclerosis

Epidemiologic studies have firmly established that plasma concentrations of HDL cholesterol are inversely correlated to the risk of developing atherosclerosis and coronary artery disease.1 Although the protective mechanism(s) is not fully understood, HDL is thought to remove unesterified cholesterol from the arterial wall and deliver it to the liver as cholesteryl ester for elimination, a process referred to as reverse cholesterol transport.2

Scavenger receptor class B type I (SR-BI), a member of the CD36 superfamily, is predominantly expressed in the liver and steroidogenic tissues, where it mediates selective uptake of cholesteryl ester from HDL.3–4 Hepatic overexpression of SR-BI in mice by means of transgenes5–7 or adenoviral vectors8–10 results in a reduction of HDL cholesterol, enhanced secretion of cholesterol into the bile, and decreased atherosclerosis. On the other hand, gene-targeted interruption of SR-BI in mice leads to the accumulation of abnormally large HDL particles11,12 impaired secretion of biliary cholesterol,13 and accelerated atherosclerosis.14 Mice deficient in both SR-BI and apolipoprotein (apo) E develop early, occlusive, atherosclerotic coronary artery diseases and die prematurely at 6 to 8 weeks of age.15,16 These data indicate that hepatic SR-BI plays an important role in the late stages of reverse cholesterol transport and protects mice against the development of atherosclerosis.

SR-BI is also expressed in macrophages, including tissue macrophages, monocyte-derived macrophages, and macrophages in atherosclerotic lesions.17,18 The expression of SR-BI in macrophages appears to be regulated by cholesterol loading,18 as well as by activators of peroxisome proliferator–activated receptors17. SR-BI has been implicated in cholesterol efflux,19 the initial step in reverse cholesterol transport. Cell culture studies have shown that SR-BI promotes net cholesterol efflux from cells when there is a favorable choles-
terol gradient\textsuperscript{20,21} and that the rate of cholesterol efflux mediated by HDL or serum is correlated with levels of cellular SR-BI expression.\textsuperscript{19–21} Taken together, these data suggest that macrophage SR-BI is also involved in the initial steps of reverse cholesterol transport and therefore might be protective against atherogenesis. However, direct evidence that macrophage SR-BI expression is antiatherogenic is lacking.

To determine whether macrophage SR-BI expression protects against atherosclerotic lesion development, we used a murine bone marrow transplantation (BMT) model in which bone marrow from SR-BI\textsuperscript{7–5} apoE\textsuperscript{7–5} mice was transplanted into recipient apoE\textsuperscript{7–5} mice to examine the impact of macrophage SR-BI expression in the development of atherosclerosis. We demonstrated that inactivation of macrophage SR-BI promotes the development of atherosclerosis in apoE-deficient mice in the absence of changes in plasma lipids, HDL subpopulations, and cholesterol efflux.

**Methods**

**Mice and Genotyping**

SR-BI\textsuperscript{19–20} mice (1:1 mixed C57BL/6\times129 genetic background) and apoE\textsuperscript{7–5} mice (C57BL/6 genetic background) were obtained from The Jackson Laboratory (Bar Harbor, Me). To generate SR-BI\textsuperscript{7–5} or SR-BI\textsuperscript{7–5} apoE\textsuperscript{7–5} mice on C57BL/6 background, SR-BI\textsuperscript{7–5} mice were mated with apoE\textsuperscript{7–5} mice, and the doubly heterozygous offspring were backcrossed with apoE\textsuperscript{7–5} mice for 7 generations or higher to produce SR-BI\textsuperscript{7–5} apoE\textsuperscript{7–5} offspring on the C57BL/6 background. SR-BI\textsuperscript{7–5} apoE\textsuperscript{7–5} mice were then mated to generate female SR-BI\textsuperscript{7–5} apoE\textsuperscript{7–5} or SR-BI\textsuperscript{7–5} apoE\textsuperscript{7–5} mice as donors for BMT. All donor mice used for BMT were 4 to 6 weeks old. Recipient, female, apoE-deficient mice (C57BL/6 genetic background and 6 weeks old) for BMT were purchased from The Jackson Laboratory.

SR-BI genotypes were determined by polymerase chain reaction (PCR) analysis of DNA extracted from tail biopsies, as described.\textsuperscript{11} ApoE genotyping was performed by a PCR protocol provided by The Jackson Laboratory.

**Animal Procedures**

All mice were maintained in microisolate cages on a rodent chow diet (No. 5010, Purina Mills, Inc) and acidified water (pH 2.8). Animal care and experimental procedures were performed according to the regulations of the Institutional Animal Care and Usage Committee of Vanderbilt University.

**Lethal Irradiation and BMT**

Lethal irradiation and BMT were performed as previously described.\textsuperscript{22} Six-week-old recipient mice were lethally irradiated with a single dose of 9 Gy by using a cobalt-60 gamma source on the day of transplantation. Bone marrow cells from the donor mice were collected as described,\textsuperscript{22} and 5.0\times10\textsuperscript{5} donor bone marrow cells were injected into each recipient mouse through the retro-orbital venous plexus 4 hours after irradiation.

**Quantification of Atherosclerotic Lesions**

Atherosclerotic lesions in the proximal aorta were quantified according to Paigen et al.\textsuperscript{23} As described in detail by our group,\textsuperscript{22} 15 consecutive cryosections of 10-mm thickness per mouse were obtained from the aortic sinus downward, starting at the position of leaflet appearance. After being stained with oil red O and counterstained in Mayer’s hematoxylin, lesion area was measured as described.\textsuperscript{22} Analysis of lesion area in en face preparations of aorta was performed as previously described.\textsuperscript{22}

**2-D Gel Electrophoresis**

Separation of plasma HDL subpopulations by agarose gel electrophoresis in the first dimension and nondenaturing polyacrylamide gradient gel electrophoresis in the second dimension was performed as described (2-D electrophoresis).\textsuperscript{24,25} After transfer of lipoproteins to nitrocellulose membranes, apoAI-containing lipoproteins were immunolocalized with rabbit anti-mouse apoAI (Biodesign). Rabbit antibody was detected with \textsuperscript{125}I-labeled anti-rabbit immunoglobulin (Zymed) prepared by the method of McFarlane.\textsuperscript{26} The radioactivity was then quantified on a phosphoimageing device (Molecular Imager FX System, Bio-Rad).

**Plasma Lipid and Lipoprotein Analyses**

All mouse plasma samples were collected after a 12-hour fast. Plasma cholesterol and triglyceride levels were measured enzymatically.\textsuperscript{22} Plasma lipoproteins were separated by fast protein liquid chromatography with Superose 6 columns (Pharmacia) in a fast protein liquid chromatography system (600E, Waters) as described.\textsuperscript{22} The lipoprotein fractions were then analyzed for cholesterol contents.

**Cell Culture and Preparation of Total RNA**

Mouse peritoneal macrophages were collected 3 days after intraperitoneal injection with 3% thioglycollate. Cells were plated in 6-well plates in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and incubated at 37°C with 5% CO\textsubscript{2}. After 4 hours, the medium was removed and replaced with DMEM containing 4% fetal bovine serum. Macrophages were cultured overnight, and total RNA was extracted from macrophages with Trizol reagent (Invitrogen) according to the manufacturer’s instructions.

**Quantification of SR-BI mRNA by Real-Time RT-PCR**

The relative quantities of SR-BI message were measured by a real-time reverse transcriptase (RT)–PCR. The primer sequences for SR-BI were as follows: 5’TCCCTCATAGCACGACGTGTT-3 (forward) and 5’TTCCACATCCGAGGACA-3 (reverse). The TaqMan probe sequence for SR-BI was 5’6FAM-CTCAAGATGTCGGCTTTGACCCGAGCT-3’ (Molecular Probes, Eugene, Ore). A commercially available kit (TaqMan 1-step RT-PCR master mix reagent kit, P/N 4309169, ABI) was used for RT-PCR. Relative quantification of SR-BI mRNA was normalized with 18S rRNA as an endogenous control. Thermal cycling conditions for SR-BI consisted of an initial RT step at 48°C for 30 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 58°C. Cycle threshold (C\textsubscript{T}) was determined according to the instructions from ABI. The data were analyzed by the comparative C\textsubscript{T} method and were confirmed by the standard curve method, as described.\textsuperscript{27}

**Isolation and Modification of Lipoproteins**

Human plasma LDL (d=1.02 to 1.063 g/mL) and HDL (d=1.063 to 1.21 g/mL) were isolated by sequential ultracentrifugation.\textsuperscript{28} Acetylated LDL (AcLDL) was prepared according to Basu et al.\textsuperscript{29} Human apoA-I was purified from human plasma HDL as described.\textsuperscript{24,25} ApoA-I discs were prepared with apoA-I and 1-palmitoyl 2-oleoyl phosphatidylcholine at a ratio of 1:100 (wt/wt), according to Sparks et al.\textsuperscript{26} and isolated by ultracentrifugation at a density of 1.063 to 1.21 g/mL.

**Cholesterol Efflux**

Cholesterol efflux from mouse peritoneal macrophages was measured as previously described.\textsuperscript{30} In brief, the cells were incubated for 48 hour with DMEM containing 100 μg/mL Ac LDL, 1% fetal bovine serum, and 6 μCi [\textsuperscript{3}H]cholesterol/mL. After an overnight equilibration in DMEM containing 0.1% bovine serum albumin, the cells were washed once with 0.5 mL of 1% bovine serum albumin in minimal essential medium (MEM) and once with MEM. For efflux, the cells were incubated for the indicated times with DMEM alone or...
SR-BI mRNA levels were expressed as relative expression, compared with expression in macrophages collected from SR-BI<sup>+/+</sup> apoE<sup>−/−</sup> → apoE<sup>−/−</sup> mice. Results presented are mean±SEM from 4 individual macrophage samples in each recipient group.

Western Blots of Murine SR-BI and ATP-Binding Cassette A1

Cellular extracts from cultured macrophages were separated by 4% to 20% Tris-glycine gels (NaPAGE, for SR-BI) or 3% to 8% Tris-acetate gels (NaPAGE, for ATP-binding cassette A1 [ABCA1]) and transferred to nitrocellulose membranes. Murine SR-BI or ABCA1 was detected with a primary antibody (Novus Biological) and visualized by chemiluminescence (ECL Plus, Amersham).

Statistical Analysis

Data were expressed as means±SEM and were compared by Student’s t test. Statistical significance was defined as P<0.05.

Results

To examine the in vivo role of macrophage SR-BI in the development of atherosclerosis, BMT was used to create apoE-deficient mice null for macrophage SR-BI expression. Six-week-old, female, apoE-deficient mice were lethally irradiated and rescued with bone marrow cells collected from female SR-BI<sup>+/+</sup> apoE<sup>−/−</sup> or SR-BI<sup>+/−</sup> apoE<sup>−/−</sup> mice. To evaluate the extent of reconstitution of recipient mice with donor marrow, mouse peritoneal macrophages from the recipient apoE-deficient mice were harvested at the time of sacrifice, and SR-BI expression was studied by a quantitative, real-time RT-PCR. As shown in Figure 1, SR-BI<sup>+/−</sup> apoE<sup>−/−</sup> → apoE<sup>−/−</sup> mice had almost no SR-BI expression compared with SR-BI<sup>+/+</sup> apoE<sup>−/−</sup> → apoE<sup>−/−</sup> mice, confirming the successful reconstitution of recipient mice with donor marrow cells. When the total RNA samples isolated from SR-BI<sup>+/+</sup> apoE<sup>−/−</sup> marrow recipient mice (n=4) were mixed with those isolated from SR-BI<sup>−/−</sup> apoE<sup>−/−</sup> recipients (n=4)

at a ratio of 1:10 for quantitative analysis of SR-BI mRNA, an average 70% reduction of SR-BI message was detected compared with the SR-BI<sup>+/+</sup> apoE<sup>−/−</sup> samples, indicating at least 90% engraftment of donor bone marrow in recipient mice (Figure 1). Furthermore, parallel BMT with ROSA-26 mice as bone marrow donors under the same conditions revealed >93% reconstitution of recipient mice with macrophages (CD11b<sup>+</sup>) of donor origin (data not shown).

Plasma lipid and lipoproteins were characterized in apoE-deficient mice before and after BMT. After transplantation, all recipient mice were maintained on a chow diet for 12 weeks. Serum cholesterol and triglyceride levels were determined after BMT at 4-week intervals. No statistically significant differences were observed in plasma cholesterol and triglyceride levels at any time point (Table). Fast protein liquid chromatography was used to examine changes in the distribution of plasma lipoproteins after BMT. Lipoprotein profiles remained unchanged between the 2 recipient groups over the course of the study, and data from 12 weeks after BMT are shown in Figure 2.

The principal goal of the current study was to evaluate the effect of inactivation of macrophage SR-BI expression on atherogenesis. Twelve weeks after transplantation, quantitative analyses of cross sections of the proximal aorta revealed an 86% increase in mean atherosclerotic lesion area in...
SR-BI+/apoE−/− mice compared with SR-BI+/+/apoE−/− mice (109.50 ± 18.08 versus 58.75 ± 9.58 × 10^3 μm^2, mean ± SEM; P = 0.017; Figure 3). En face analysis of pinned-out aortas demonstrated an 18% increase in atherosclerosis in SR-BI+/−/apoE−/− mice compared with SR-BI+/+/apoE−/− mice, but it did not reach statistical significance (P = 0.08; Figure 3).

The impact of hepatic expression of SR-BI on HDL metabolism has been examined by several groups. However, the role of macrophage SR-BI in HDL metabolism is unclear. By 2-D electrophoresis, we investigated the effect of macrophage SR-BI expression on plasma HDL subpopulations. No significant differences in HDL subpopulations were detected 12 weeks after BMT (Figure 4).

To examine the hypothesis that SR-BI deficiency in macrophages leads to diminished efflux of cholesterol to HDL, cholesterol efflux studies were performed on peritoneal macrophages from apoE−/−, SR-BI−/− apoE−/−, SR-BI+/−, and C57BL/6 mice, with human plasma HDL and apoA-I discs as acceptors. Surprisingly, no significant differences in cholesterol efflux to HDL or apoA-I discs were found among the 4 types of macrophages (Figure 5).

To verify that apoE-deficient macrophages do express SR-BI protein, peritoneal macrophages from apoE-deficient mice were isolated, and cell lysates were prepared after 2-hour culture and after incubation with AcLDL for 48 hours (right before cholesterol efflux), respectively. Western blots with anti-SR-BI antibody demonstrated that SR-BI+/+ apoE−/− macrophages expressed abundant SR-BI protein, whereas SR-BI−/− apoE−/− macrophages had no detectable SR-BI protein (Figure 6A). The expression of ABCA1, another cell-surface protein that mediates cholesterol efflux,
death, suggesting that hepatic expression of SR-BI is coronary atherosclerosis, myocardial infarction, and early develop severe hypercholesterolemia, accelerated occlusive sharp contrast to apoE/SR-BI double-knockout mice, which SR-BI did not appear to have increased mortality. This is in a similar trend in en face analysis of aortas (Figure 3). These results strongly support an antiatherogenic role for macrophage SR-BI expression in vivo.

The increase in atherosclerosis in apoE-deficient mice due to loss of macrophage SR-BI expression occurred in the absence of changes in plasma lipid and lipoprotein profiles. After BMT, the plasma lipid levels over the course of 12 weeks were similar between the 2 groups of recipient mice (Table). Plasma lipoprotein profiles analyzed by fast protein liquid chromatography (Figure 2) in the 2 groups of recipient mice were also similar, indicating that macrophage SR-BI expression has no significant effect on plasma lipids or lipoprotein distributions.

The extent of lesion area in the en face analysis of aortas of SR-BI+/−/apoE−/− mice was greater than that of SR-BI+/−/apoE−/− mice (Figure 3), but it was not statistically significant. It should be noted that the total lesion areas in en face preparation were small, <1%, indicative of early atherosclerotic lesions. In mice, atherosclerotic lesions develop first in the proximal aorta and then progress distally. Although there was a strong trend for an increase in lesion area in the en face analysis of the experimental group, the very low lesion burden in the en face analysis might have limited our ability to detect a significant difference by this approach.

The apoE-deficient mice with inactivation of macrophage SR-BI did not appear to have increased mortality. This is in sharp contrast to apoE/SR-BI double-knockout mice, which develop severe hypercholesterolemia, accelerated occlusive coronary atherosclerosis, myocardial infarction, and early death, suggesting that hepatic expression of SR-BI is essential for survival of apoE-deficient mice. Furthermore, our results indicate that a combined deficiency of SR-BI and apoE in bone marrow–derived cells is insufficient to reproduce the lethal phenotype seen in SR-BI+/−/apoE−/− mice.

Cell culture studies established that cellular cholesterol efflux to HDL is correlated with the level of SR-BI expression, which leads to the hypothesis that SR-BI–facilitated cholesterol efflux might be an important mechanism under pathophysiologic conditions to maintain cellular cholesterol homeostasis. This hypothesis is supported by the observations that atherosclerotic lesion macrophages express SR-BI and that SR-BI expression in tissue macrophages is upregulated by cholesterol loading. However, our efflux study with HDL or apoA-I discs as acceptors did not show differences in cholesterol efflux in AcLDL–loaded macrophages with and without SR-BI expression. We also did not find any difference in ABCA1 expression in macrophages with or without SR-BI expression. It is intriguing that SR-BI deficiency does not cause an upregulation in ABCA1 expression in macrophages, leading to enhanced efflux via this pathway. Our data appear to suggest that the antiatherogenic mechanism of macrophage SR-BI might not relate to its ability to efflux cholesterol from macrophages. It is possible that lesional macrophages might express more SR-BI than do peritoneal macrophages, as Ji et al have shown an increased SR-BI expression in lesional macrophages in apoE-deficient mice. Alternatively, SR-BI deficiency in macrophages might result in the accumulation of cholesterol in an intracellular pool that is inaccessible to cholesterol efflux.

Macrophage SR-BI might influence atherogenesis by mechanisms other than cholesterol efflux. It is possible that inactivation of SR-BI in macrophages might alter the expression of genes in these cells that are involved in atherogenesis. Frank et al reported that expression of SR-BI dramatically increases the stabilization of caveolin-1, a characteristic protein found in plasma membrane invaginations called caveolae. Because caveolae regulate cellular cholesterol homeostasis and participate in cell signaling and transcytosis, loss of SR-BI expression in macrophages could affect certain signal transduction pathways, cellular cholesterol homeostasis, and ultimately, the development of atherosclerosis. Recently, Van Eck et al demonstrated that SR-BI deficiency results in significant induction of genes implicated in adhesion and transendothelial migration of monocytes in arterial walls, indicative of an enhanced inflammatory response in the arterial wall in SR-BI–deficient mice.

SR-BI plays a pivotal role in HDL metabolism. Early work by Krieger’s group and others has established that hepatic expression of SR-BI determines plasma HDL levels and influences HDL subpopulations. However, little is known about the impact of macrophage SR-BI expression on plasma HDL subfractions. In this study, we used 2-D gel electrophoresis to determine whether the expression of macrophage SR-BI alters HDL subfractions. As presented in Figure 4, no significant changes in HDL subpopulations after BMT over a course of 12 weeks were observed, suggesting that macrophage SR-BI does not exert a detectable effect on HDL subpopulations. This observation is in line with the finding of Haghpassand et al, that macrophage ABCA1 has minimal contribution to plasma HDL levels. This might be explained by the fact that the number of macrophages in the body is relatively small compared with the total number of cells and therefore, insufficient to influence HDL subpopulations.

In summary, apoE-deficient mice with selective inactivation of macrophage SR-BI were created by transplanting lethally irradiated, female, apoE-deficient mice with bone marrow cells collected from SR-BI+/−/apoE−/− mice. Twelve weeks after BMT, SR-BI+/−/apoE−/− mice fed a
chow diet developed significantly more atherosclerosis in the proximal aorta than did SR-BI−/− apoE−/− mice, in the absence of significant differences in plasma lipids, lipoprotein profiles, HDL subpopulations, and cholesterol efflux. We conclude that the expression of macrophage SR-BI is protective against the development of atherosclerosis in vivo and that the antiatherogenic mechanism(s) of macrophage SR-BI might not derive from its ability to efflux cholesterol. Further investigations of the role of macrophage SR-BI in inflammation and cellular cholesterol homeostasis will increase our understanding of the antiatherogenic mechanism(s) of macrophage SR-BI expression. Macrophage SR-BI provides a new therapeutic target for the design of strategies to prevent and treat atherosclerosis.

**Note Added in Proof**

Covey et al. have recently reported that SR-BI expression in bone marrow-derived cells contributes to protection from atherosclerosis in LDLR-deficient mice.

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**References**


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