Expression of Human Scavenger Receptor Class B Type I in Cultured Human Monocyte-Derived Macrophages and Atherosclerotic Lesions

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Abstract—The scavenger receptor class B type I (SR-BI) and its human homologue CLA-1 (CD36 and LIMPII Analogous-1) have recently been identified to bind HDL and mediate the selective uptake of HDL lipids. Tissue distribution of both murine and human receptors is quite similar, in that they are expressed abundantly in liver and steroidogenic tissues. However, expression and function of the human SR-BI (hSR-BI), in the periphery of reverse cholesterol transport such as macrophages, are still unclear. In the present study, we have raised two different kinds of anti–hSR-BI polypeptide antibodies (Abs): one against the extracellular domain and the other against the intracellular domain. We have investigated the expression of hSR-BI mRNA and immunoreactive mass in freshly isolated cultured human monocyte-derived macrophages (hMφ) and in atherosclerotic lesions. Contrary to the earlier report, hSR-BI mRNA was expressed in cultured hMφ and markedly upregulated with differentiation, determined by Northern blot and reverse transcriptase–based polymerase chain reaction analyses. The mRNA expression pattern during differentiation of hMφ was very similar to those of SR class A and another member of SR class B, CD36. Protein expression was confirmed by Western blot analyses with the above Abs to show a major 83-kDa band. Modified lipoproteins such as oxidized LDL and acetylated LDL induced a 5-fold increase in mRNA and protein expression of hSR-BI. Confocal immunofluorescence microscopy demonstrated that hSR-BI immunoreactive mass was detectable as a heterogeneous, punctate staining pattern. Furthermore, immunohistochemical analysis showed that immunoreactive mass of hSR-BI was detected in foam cells in human aortic atherosclerotic lesions and that there was no significant difference of staining patterns between the two Abs. This study clearly demonstrates that hSR-BI is expressed in the lipid-laden macrophages in human atherosclerotic lesions, suggesting that it is very important to know its function and regulation in hMφ to understand the biological utility of this molecule.

Key Words: atherosclerosis ■ human scavenger receptor class B type I ■ high-density lipoprotein ■ reverse cholesterol transport ■ scavenger receptor

High-density lipoprotein (HDL) is postulated to have antiatherogenic functions. One of the major protective systems against atherosclerosis is called reverse cholesterol transport (RCT), in which HDL plays a crucial role as a shuttle carrying cholesterol derived from peripheral tissue to the liver. In the first step of RCT, HDL removes cholesterol from atherosclerotic lesions. The cholesterol incorporated into HDL particles is esterified by the action of lecithin:cholesterol acyltransferase (LCAT). The cholesteryl ester in HDL is transferred from HDL to the apolipoprotein (apo) B–containing lipoproteins by plasma cholesteryl ester transfer protein (CETP). In addition to LCAT and CETP, hepatic triglyceride lipase (HTGL) and lipoprotein lipase (LPL) are thought to take some part in the RCT system by modulating the particle size and composition of HDL. Apo B–containing lipoproteins and HDL particles are finally taken up by the liver and steroidogenic tissues, the terminal of RCT.

Recently, scavenger receptor class B type I (SR-BI) and its human homologue CLA-1 (CD36 and LIMPII Analogous-1) have been cloned and identified to bind HDL and mediate selective uptake of HDL lipids. Studies with genetically engineered mice have demonstrated that SR-BI has an essential role in taking up cholesterol in liver and steroidogenic tissues and have suggested that levels of SR-BI expression may determine the plasma HDL cholesterol levels in rodents. However, expression and function of SR-BI and its human homologue, especially in the initial step of RCT, such
We have examined the expression of hSR-BI in freshly isolated hMφ and in the human atherosclerotic lesion. In the present study, we have raised two different kinds of anti–hSR-BI polypeptide antibodies: one against the extracellular domain and the other against the intracellular domain. We have examined the expression of hSR-BI in freshly isolated hMφ. We clearly demonstrate that hSR-BI is expressed in both cultured hMφ and in the human atherosclerotic lesion.

Materials and Methods

**Human Monocyte-Derived Macrophages and Tissue Specimens**

Mononuclear cells were isolated from healthy volunteers by density gradient centrifugation using lymphocyte separation solution (Nacalai Tesque, Kyoto, Japan) and were suspended in RPMI-1640 medium supplemented with 10% human type AB serum and antibiotics. In the indicated experiments, we used 10% FCS in place of 10% human AB serum. Mononuclear cells were plated in 10-cm cell culture dishes (Falcon Labware; Becton-Dickinson) and incubated in culture medium. The medium was replaced every 3 days. Cell viability determined by trypan blue exclusion was 95% in all experiments.

Human aortic tissues (mainly descending thoracic aorta) were obtained from 20 autopsy cases with informed consents from the families. Tissues were embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co, Ltd, Tokyo, Japan), frozen in liquid nitrogen, and stored at −80°C until use. Cryostat sections were 6 μm thick and air-dried for 30 minutes.

**Isolation and Modification of LDL**

LDL (1.019 < density < 1.063 g/mL) was isolated from the sera of normolipidemic healthy volunteers by preparative ultracentrifugation. Acetylated LDL (AcLDL) was made by repeated additions of acetic anhydride. Oxidized LDL (OxLDL) was prepared by incubating LDL with 5 μmol/L CuSO₄ for 24 hours at 37°C. Modified LDLs used were extensively dialyzed against PBS and sterilized before use. The extent of oxidative modification of LDL was evaluated by agarose gel electrophoresis. We have measured the endotoxin concentration in the OxLDL preparation by using an ELISA kit and the concentration was <0.5 pg/mL, which is below the level that has any effect on biological activity.

**Cloning of hSR-BI cDNA from hMφ and Transient Transfection of hSR-BI cDNA in Chinese Hamster Ovary (CHO) Cells**

Total RNA from hMφ on the seventh day after plating was used for reverse transcriptase–based polymerase chain reaction (RT-PCR). Two micrograms of total RNA was reverse-transcribed by SuperScript Reverse Transcriptase (GibcoBRL), according to the manufacturer’s recommendation. One tenth of the volume of RT reactions was applied for the PCR with primers P1 and P2 by Advantage cDNA synthesis kit (Clontech). PCR products with expected length were subcloned into pGEM 3zf(−) (Invitrogen). The entire insert was sequenced by an autosequence analyzer (Perkin-Elmer), according to the manufacturer’s recommendation. The sequence obtained was completely identical to the sequence of the long form of hSR-BI (GenBank accession No. Z22555). The full-length cDNA was cloned into a mammalian expression vector, pcDNA 3.1(−) (Invitrogen) and transfected into CHO cells by Lipofectamine reagent (GibcoBRL).

**Antibodies Against hSR-BI**

Two different kinds of rabbit polyclonal antipolypeptide antibodies were raised against amino acid residues 75 to 93 and 490 to 509 of as macrophages in blood vessels, are still not clear and rather controversial. Some studies with in situ hybridization technique showed that murine SR-BI mRNA is expressed in the atherosclerotic lesions in atherogenic-engineered mice. One earlier report as to human SR-BI (hSR-BI) showed that hSR-BI expression was much lower in human monocyte-derived macrophages (hMφ) than in a human promonocytic leukemia cell line, THP-1 and that the expression was decreased after stimulation by phorbol 12-myristate 13-acetate (PMA). On the other hand, SR-BI belongs to the family of SR class B that includes CD36 and lysosomal integral membrane protein-II (LIMPII). We have reported that CD36 is an important receptor for oxidized LDL by the study of subjects with genetic CD36 deficiency and that CD 36 is expressed with genetic CD36 deficiency and that CD 36 is expressed in lipid-laden macrophages in human aortic and coronary atherosclerosis by morphological studies (Nakagawa Y et al, unpublished data, 1999).

In the present study, we have raised two different kinds of anti–hSR-BI polypeptide antibodies: one against the extracellular domain and the other against the intracellular domain. We have examined the expression of hSR-BI in freshly isolated hMφ. We clearly demonstrate that hSR-BI is expressed in both cultured hMφ and in the human atherosclerotic lesion.
the long form of hSR-BI sequence (GenBank accession No. Z22555) (Sawady Technology, Tokyo, Japan). Their specificities and titers were determined by analysis of reactivities to the synthetic peptides used for immunization. ELISA analysis showed high-titer immuno-reactivity of the antisera to the peptide for immunization.

**Western Blot Analysis**

Human monocyte-derived macrophages were lysed and passed 10 times through 25-gauge needles. Human liver and kidney homogenates were purchased (Clontech). Samples were reduced with 2-mercaptoethanol in gel loading buffer, fractionated in 5% to 10% Tris-glycine gel (BioRad), and transferred to a 0.45-mmol/L polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked with a solution of 0.1% Tween 20/PBS (PBS-T) containing 1% BSA overnight and then incubated with rabbit anti-hSR-BI polyclonal antibodies at 2 μg/mL in PBS-T/BSA for 1 hour at room temperature followed by washing 3 times for 5 minutes with PBS-T buffer. The blot was reblocked with PBS-T/BSA for an additional hour before adding HRP conjugated with goat anti-rabbit immunoglobulin (IgG) (1:500 dilution) for another hour at room temperature. After washing 3 times for 10 minutes with PBS-T, detection was performed by the protocol of the enhanced chemiluminescence (ECL) plus kit (Amersham).

**RNA Isolation and Northern Blot Analysis**

Total cellular RNA from hMø was isolated using Trizol reagent (Gibco BRL). For Northern analysis, 10 μg RNA was separated on 1.2% agarose gels and transferred onto positively charged nylon membranes by the Northern Max kit (Ambion). Biotinylated hSR-BI and GAPDH cRNA probes were made by in vitro transcription by the StripEZ kit (Ambion) and labeled by the Psoralen-Biotin kit (Ambion). Hybridization and visualization were performed, using the BioDetect kit (Ambion). Data were analyzed by the National Institutes of Health (NIH, Bethesda, Md) image programs.

**Reverse Transcription–Coupled Polymerase Chain Reaction (RT-PCR)**

Conditions for RT-PCR used 2 μg of total RNA and used downstream priming with primers P3, P4, P5, and P6 in the presence of 50 U Superscript reverse transcriptase (GibcoBRL) at 42°C for 60
minutes. Fragments of 696 bp, 443 bp, and 330 bp from hSR-BI, CD36, and SR-A, respectively, were amplified from cDNA samples in the same tube. A 244-bp fragment from human GAPDH was amplified in a separate tube. Sample sizes and the number of amplification cycles were optimized to produce measurements within a linear range. After heating the reaction at 75°C for 5 minutes, one tenth of the volume was used for PCR with Advantage cDNA Polymerase Mix (Clontech). After heat denaturation at 95°C for 5 minutes, the following parameters were used: 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 1 minute. Cycle number was 28 for hSR-BI, CD36, and SR-A and 20 for GAPDH. The density was quantitated using NIH image programs.

Immunofluorescent Microscopy of Macrophages

Human monocyte-derived macrophages were washed with PBS, fixed for 10 minutes with paraformaldehyde on ice, and permeabilized for 5 minutes with 0.2% Triton X-100. Cells were blocked with PBS containing 10% FBS and 10% human immunoglobulins to inhibit the interaction of antibodies with fragment-crystallizable receptors and to decrease the nonspecific bindings. Primary antibodies (1:100 dilution) were applied for 1 hour. After washing twice, rhodamine phalloidin (Molecular Probes) and FITC-conjugated anti-rabbit IgG were added to visualize F-actin and hSR-BI, respectively. Images were acquired successively for each fluorescent probe by confocal laser microscopy as a single 1-μm-thick optical section using a Zeiss LSM 410 confocal microscope and ×60 objective.

Immunohistochemical Analysis

Frozen sections were fixed for 10 minutes with cold acetone and washed in 0.05 mol/L Tris-HCl buffer (pH 7.6) for 10 minutes. Endogenous peroxidase activity was quenched by incubating the section for 10 minutes with 3% hydrogen peroxide. After washing twice, rhodamine phalloidin (Molecular Probes) and FITC-conjugated anti-rabbit IgG were added to visualize F-actin and hSR-BI, respectively. Images were acquired successively for each fluorescent probe by confocal laser microscopy as a single 1-μm-thick optical section using a Zeiss LSM 410 confocal microscope and ×60 objective.


Figure 3. Expression of SR mRNA in in vitro–differentiated human macrophages, determined by RT-PCR. Total RNA was extracted from human hMφ cultured in 10% AB serum/RPMI at the indicated days after plating. A, Agarose gel electrophoresis of RT-PCR products. Two micrograms of total RNA was subjected into RT reaction with Superscript Reverse Transcriptase at 42°C for 1 hour. After heating the RT reaction at 75°C for 10 minutes, one tenth of the volume was used for PCR with Advantage cDNA Polymerase Mix (Clontech). After heat denaturation at 95°C for 5 minutes, the following parameters were used: 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 1 minute. Cycle number was 28 for hSR-BI, CD36, and SR-A and 20 for GAPDH. B, Relative amounts of expression were calculated by NIH image programs.

Primers Used in the Present Study

P1: 5′-ATA AGC TTC TAC AGT TTT GCT TCC TGC AG-3′, hSR-BI cDNA nucleotides 1599 to 1579 (GenBank accession No. Z22555)
P2: 5′-ATG GTA CCA TGG GCT GCT CCG CCA AAG CG-3′, hSR-BI cDNA nucleotides 70 to 90 (GenBank accession No. Z22555)
P3: 5′-TGA CCG GGT GGA TGT CCA GGA AC-3′, hSR-BI cDNA nucleotides 1201 to 1179 (GenBank accession No. Z22555)
P4: 5′-ATT CCC ATG TTC TTT TTT GCT TCC TGC AG-3′, hSR-BI cDNA nucleotides 559 to 538 (GenBank accession No. E05210)
P5: 5'-GGC TGC ATC TGT ACC ATT AAT CAT GT-3', CD36 cDNA nucleotides 880 to 854 (GenBank accession No. L06850)
P6: 5'-GGC AGT GAT GGC ATG GAC TGT G-3', GAPDH cDNA nucleotides 609 to 588 (GenBank accession No. M33197)
P7: 5'-TGA TGA TGG AGA ATA AGC CCA T-3', hSR-BI cDNA nucleotides 506 to 527 (GenBank accession No. Z22555)
P8: 5'-GCA GTT CTC CTA GCT CTC ATT GGA-3', SR-A cDNA nucleotides 230 to 253 (GenBank accession No. E05210)
P9: 5'-TGT AAC CCA CGC TGA GGA C-3', CD36 cDNA nucleotides 438 to 459 (GenBank accession No. L06850)
P10: 5'-CAC CAC CAT GGA GAA GGC TGG-3', GAPDH cDNA nucleotides 366 to 386 (GenBank accession No. M33197)

Results
Characterization of Two Kinds of Anti–hSR-BI Polypeptide Antibodies
To identify the hSR-BI protein expression in hMφ, we have raised two different kinds of anti–hSR-BI polypeptide anti-
bodies: one against the extracellular domain (AA 75 to 93, GenBank accession No. Z22555) of hSR-BI polypeptide, designated hSR-BI-75, and the other against the intracellular domain (AA 490 to 509), designated hSR-BI-490. Figure 1 shows the data of Western blot analysis with the two antibodies raised, demonstrating that whole cell lysates from hSR-BI-transfected CHO cells as well as human liver homogenates showed a major 83-kDa band. Patterns of immunoreactive mass detected by the Abs were quite similar.

Expression of hSR-BI mRNA and Protein in Cultured hMφ

We have investigated the expression of SR-BI of hMφ in 10% human AB serum. As shown in Figure 2A and 2B, Northern blot analysis using a 550-nt (nucleotides 1050 to 1599, hSR-BI cDNA, GenBank accession No. Z22555) biotinylated hSR-BI cRNA probe showed a major 2.9-kbp mRNA species. The size of the transcript was consistent with that of previous reports. However, contrary to the earlier report,13 expression levels were markedly upregulated to 80-fold during differentiation of monocytes to macrophages. Figure 2C shows the data of Western blot analysis using hSR-BI-490 antibody for in vitro differentiated hMφ. During the differentiation, immunoreactive mass of hSR-BI was increased after plating in parallel with the increased expression of hSR-BI mRNA. To investigate the discrepancy between our findings and the earlier report by Murao et al,13 we have examined the effect of FCS on the expression of hSR-BI, because they used 10% FCS in place of 10% AB serum. RT-PCR showed a differentiation-dependent increase of SR-BI expression in macrophages cultured, even in medium containing 10% FCS, as shown in Figure 2D.

In Vitro Differentiation Induces Expression of SRs in Cultured hMφ

To compare the expression pattern of hSR-BI with those of other SRs, such as SR class A and another member of SR class B, CD36, we have performed RT-PCR analyses. As shown in Figure 3, other SRs, CD36 and SR-A, were also markedly upregulated during the in vitro differentiation in medium containing 10% human AB serum. On day 0, a trace amount of hSR-BI immunoreactive mass was detectable, as shown in Figure 2C, whereas mRNA expression was hardly seen by Northern blot and RT-PCR analyses (Figures 2A and 3A). We have done the second round of RT-PCR with nested primers and successfully amplified the expected length of cDNA and confirmed the entire sequence (data not shown).

Modified Lipoproteins Induce the Expression of hSR-BI In Vitro

We have investigated whether modified lipoproteins such as OxLDL and AcLDL induce the expression of hSR-BI. The addition of 50 μg/mL of protein of each modified LDL for 24 hours made ≈5-fold increase in both mRNA and protein of hSR-BI, as shown in Figure 4A and 4B.

Figure 5. Detection of hSR-BI protein in hMφ by confocal laser microscopy. Macrophages cultured for 7 days were fixed and permeabilized with 3.7% formaldehyde and Triton X-100 and then incubated with indicated 1:200 rabbit anti–hSR-BI IgG. After washing by PBS, rhodamine phalloidin and FITC-conjugated anti-rabbit IgG were added to visualize F-actin (B, E, and H) and hSR-BI (A, D, and G), respectively. All images were acquired successively for each fluorescent probe by confocal laser microscopy as a single 1-μm-thick optical section using a Zeiss LSM 410 confocal microscope and a ×60 objective. Panels C, F, and I show color overlays of the hSR-BI (green) and F-actin (red) patterns.
Immunofluorescence Microscopy of hSR-BI in Cultured hMφ

Both of the anti–hSR-BI polypeptide antibodies hSR-BI-75 and hSR-BI-490 showed the heterogeneous and punctate pattern, as shown in Figure 5. As shown in Figure 6, the addition of OxLDL (50 μg/mL) into medium clearly enhanced the signals for hSR-BI along with the accumulation of lipids stained by Oil Red O.

Immunohistochemical Analysis of hSR-BI in Human Atherosclerotic Lesion of Aorta

It has not been clarified whether hSR-BI is expressed in human aortic tissues. Figure 7 shows the immunohistochemical staining of aortic specimens, demonstrating the presence of hSR-BI immunoreactive mass in lipid-laden cells in the atherosclerotic lesions. No reaction product was detectable with preimmune serum. A comparison of the staining pattern for macrophage-specific marker confirmed that hSR-BI was expressed in lipid-laden macrophages (data not shown).

Discussion

The present study demonstrates for the first time that hSR-BI is expressed and markedly upregulated in differentiated hMφ and in human atherosclerotic lesions of aorta. Our results showing the expression of hSR-BI in differentiated hMφ are in sharp contrast to an earlier study by Murao et al., who...
reported the differentiation-dependent decrease in hSR-BI expression in hMφ. We have tested more than 15 lines of hMφ from healthy volunteers, all of which showed a differentiation-dependent increase in hSR-BI mRNA. Because we have noticed the difference of culture conditions between our study and the previous one in which 10% FCS was used for culture,\(^1\)\(^3\) the expression of hSR-BI in medium containing 10% FCS was also examined. We have observed the differentiation-dependent increase of hSR-BI expression even in their condition, which indicated that the difference of sera in culture could not explain the discrepancy. We were able to demonstrate that the immunoreactive mass of hSR-BI was detected in differentiated hMφ and in human atherosclerotic lesions by two different antibodies raised. Ji et al\(^18\) reported that SR-BI mRNA was expressed abundantly in murine atherosclerotic lesions. These observations strongly suggest that SR-BIs are expressed in atherosclerotic lesions.

Recently, data have been accumulated to show that SR-BI may play an important role in both cholesterol efflux and influx. This seems to depend on the concentration gradient of cholesterol across the cell membrane. The function of SR-BI in the liver and steroidogenic tissues has been extensively analyzed by studies with genetic-engineered mice technology and cellular biology. These studies showed that SR-BI is essential for the selective uptake of HDL lipids and that the levels of expression in the liver may determine the plasma HDL cholesterol levels in rodents. However, the function of SR-BI in the blood vessels was still obscure. Some studies suggested that expression levels of SR-BI are correlated to the degree of cholesterol efflux.\(^16\) In contrast, another study showed that stable cell lines of hSR-BI had the accumulation of intracellular cholesterol.\(^13\) Our preliminary results show that the immunostaining pattern of the Mφ in the atherosclerotic lesions and Kupffer cells in human liver is different from that of hepatic parenchymal cells (Nakagawa Y, Hirano K, unpublished observation, 1998), suggesting that subcellular localization of hSR-BI is different between these cell types. Recently, Fluiter et al\(^25\) have reported that the regulation of SR-BI expression in hepatic parenchymal cells, by estradiol administration and cholesterol feeding, was opposite to that in Kupffer cells. The possibility has become focused that overexpression of SR-BI may be a new strategy for antilipidemic and antiatherosclerotic treatment, and it has recently been reported that the introduction of SR-BI into the liver reduced atherosclerosis in heterozygous LDL receptor knockout mice.\(^26\) The present study indicates that it is essential to clarify the function and regulation of hSR-BI expressed in phagocytic cells as well as hepatic parenchymal cells and steroidogenic cells to understand the biological utility of this molecule.

We have reported the molecular and biochemical bases of patients with marked hyperalophilipoproteinemia (HALP), such as a genetic cholesteryl ester transfer protein (CETP) deficiency,\(^27\) suggesting that marked HALP is not always a beneficial state but rather an impairment of the RCT system.\(^29\)\(^30\) Recently, we have found that marked HALP caused by CETP deficiency is not a longevity syndrome, on the basis of a large population-based study.\(^31\) Human genetic CETP deficiency is characterized by the accumulation of large HDL particles in plasma.\(^32\) which is also observed in SR-BI knockout mice.\(^14\) Considering that genetic defects in large percentages of patients with HALP are still unknown, it is of great importance to further analyze the function of this type of SR in hMφ and to know how relevant this molecule is in humans.

References


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