Expression of Human Scavenger Receptor B1 on and in Human Platelets

Hitomi Imachi, Koji Murao, Wenming Cao, Satoshi Tada, Tomohiko Taminato, Norman C.W. Wong, Jiro Takahara, Toshihiko Ishida

Objective—The abundance of HDL particles correlates inversely with the incidence of coronary heart disease. The human scavenger receptor B1 (hSR-B1/CLA-1) is a receptor for HDL. Expression of hSR-B1/CLA-1 mRNA and protein in human platelets was determined using reverse transcriptase–polymerase chain reaction and Western blot, respectively. Presence of the protein on the surface of platelets was shown using flow cytometry.

Methods and Results—Immunohistochemical staining for hSR-B1/CLA-1 showed that it was expressed in megakaryocytes, the platelet precursors of human bone marrow. These findings prompted us to ask whether hSR-B1/CLA-1 was differentially expressed on platelets obtained from patients with atherosclerotic disease compared with those in control subjects. Our findings showed that abundance of hSR-B1/CLA-1 was significantly reduced on the surface of platelets from patients with atherosclerotic disease. The reduced levels of hSR-B1/CLA-1 were associated with increased cholesterol ester content in platelets from patients with atherosclerotic disease compared with control subjects. A negative correlation existed between hSR-B1/CLA-1 expression and platelet aggregation. In summary, our studies show that the HDL receptor hSR-B1/CLA-1 is expressed in platelets and their precursor, the megakaryocyte. The levels of hSR-B1/CLA-1 expression correlate inversely with cholesterol ester content and platelet aggregation.

Conclusion—These findings suggest that determining the level of hSR-B1/CLA-1 expression on the platelets may be a useful clinical marker for atherosclerotic diseases. (Arterioscler Thromb Vasc Biol. 2003;23:898-904.)

Key Words: CLA-1 ■ scavenger receptor B1 ■ platelet ■ reverse cholesterol transport ■ HDL
was amplified from human monocyte-derived THP-1 cells (American Type Culture Collection) cDNA using polymerase chain reaction (PCR). The product of this reaction was inserted into a pGEX-2T vector (Pharmacia). The nucleotide sequence was verified and the peptide was expressed in Escherichia coli. The resulting fusion peptide fused to GST was isolated using glutathione-Sepharose 4B beads (Pharmacia). The bound material was used to generate an antiserum in guinea pigs. The IgG fraction from immunized animals was purified before use in Western blot and FACs analyses. Western blot analysis of proteins extracted from the cells stably expressing CLA-1 showed that the antibody directed against an extracellular portion of the protein recognized a single band with an estimated molecular mass of 83 kDa, as previously described. A second antibody, either an HRP-conjugated or a FITC-conjugated goat anti-guinea pig IgG (Sigma), was used in Western blot or flow cytometric analysis, respectively.

**Cell Culture**

HepG2 cells (obtained from RIKEN CELL BANK, Ibaragi, Japan) were grown in DMEM (Life Technologies) with 10% FCS. Mo7e cells were grown in RPMI 1640 medium (Life Technologies) supplemented with 20% FCS and 5 ng/mL human recombinant GM-CSF (Life Technologies) in a humidified atmosphere containing 5% CO₂. Human embryonic kidney HEK 293 cells (American Type Culture Collection) were cultured in DMEM with 10% FCS. HEK 293 cells were transfected with 5 µg of linearized plasmid DNA including hSR-B1/CLA1 cDNA. Stable transfectants were selected by their resistance to G418 sulfate (0.8 mg of active drug per mL), as described previously.

**Isolation of Human Platelets**

Platelets were isolated from 11 age- and sex-matched healthy volunteers (average age 57.5 ± 7.4; 6 male, 5 female; none of the controls were taking medication) and 11 patients with atherosclerotic disease, including cerebral infarction, ischemic heart disease, and arteriosclerosis obliterans (Table). The study was approved by the institutional review board of the Kagawa Medical University, and informed consent was obtained from all participants before sample collection. Blood samples were collected by venipuncture into plastic tubes containing the anticoagulant acid-citrate-dextrose buffer/sodium EDTA. The platelet-rich plasma was fractionated by centrifugation at 1000 g for 10 minutes at room temperature. The platelets were isolated by additional centrifugation of the platelet-rich plasma at 2000 g for 10 minutes, and the pellet was washed twice with PBS at 4°C.

**Amplification of CLA-1 cDNA**

Total RNA was extracted from isolated platelets or HepG2 cells using a single-step acid guanidium thiocyanate-phenol-chloroform technique, as described previously. The primer sequences for RT-PCR amplification of hSR-B1/CLA-1 mRNA were sense primer 5'-ATGATCGTGATGGTGCCGTC-3' and antisense primer 5'-ACTGAACTGGAGGTGTA-3'. Reverse transcriptase (RT)-PCR amplification of β-actin mRNA was analyzed under identical conditions using the appropriate set of primers. CLA-1 expression was determined by PCR analysis of the reverse-transcribed RNA, as described previously.

**Western Blot Analysis**

Cells were washed in PBS and lysed in PIRA buffer (10 mmol/L Tris-HCl [pH 7.4], 1% NP40, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 mol/L NaCl, 1 mmol/L EDTA, and 10 µg/mL aprotinin). The proteins were resuspended under reducing conditions, and 15 µg was fractionated by size on 7.5% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes for immunoblotting. The membranes were blocked overnight at room temperature with 0.1% Tween 20 in PBS (PBS-T) containing anti-hSR-B1/CLA-1 antibody (diluted 1/3000 from whole antiserum) or anti-cycliphilin A antibody (Biomol Research, Plymouth Meeting, Pa; diluted 1/1000). These membranes were washed with PBS-T, incubated for 1 hour at room temperature in PBS-T containing horseradish peroxidase–linked anti–guinea pig IgG (Sigma) (diluted 1/3000), and rinsed in PBS-T, and antibody binding was visualized by chemiluminescence detection (ECL, Amersham Corp).

**Immunolocalization**

Bone marrow was aspirated at the posterior iliac crest from healthy volunteers under local anesthesis using a protocol approved by the institutional review board of the Kagawa Medical University. The cellular component of the sample was obtained by centrifugation, and the cells imbedded in a paraffin block. The slices from the block were fixed in 4% paraformaldehyde, deparaffinized in xylene, and then rehydrated through graded ethanol solutions. To inhibit endogenous peroxidase activity, the processed slices were incubated at room temperature for 15 minutes with methanol containing 3% hydrogen peroxide. Sections were blocked for 60 minutes in 10% normal goat serum (NGS) in PBS and incubated for 2 hours with a guinea pig antibody directed against hSR-B1/CLA-1 (diluted 1/3000 from whole antiserum) or anti-cycliphilin A antibody (Biomol Research, Plymouth Meeting, Pa; diluted 1/1000). These slices were washed with PBS-T, and antibody binding was visualized by chemiluminescence detection (ECL, Amersham Corp).
and incubated with an avidin-biotinylated peroxidase complex (Vectorstain Elite Kit) in PBS as suggested by the manufacturer. Antibody binding was visualized with the diaminobenzidine reaction, and sections were counterstained with Mayer’s hematoxylin.

Flow Cytometric Analysis

The washed platelets were resuspended in FACS buffer (0.1% BSA in PBS) at a concentration of $1 \times 10^7$ platelets/mL in the same buffer. These cells were incubated with anti-SR-B1/CLA-1 guinea pig antibody (5:100 dilution) on ice for 1 hour followed by exposure to fluoresce in isothiocyanate (FITC)-conjugated anti–guinea pig IgG antibody (2:100 dilution, IMMUNOTECH, Marseille, France) on ice for 1 hour. Cells were then washed twice with analysis buffer and resuspended in the same buffer. For controls, cells were incubated with FITC-conjugated mouse IgG (DAKO Japan Co. Ltd, Kyoto, Japan). Platelets were also incubated with phycoerythrin (PE) conjugated anti-CD41 antibody (DAKO). Fluorescent-positive cells were analyzed on a FACSscan (Becton Dickinson).

Platelet Aggregometry

Venous blood was collected in tubes containing sodium citrate and then centrifuged at 1500g for 10 minutes to obtain platelet-rich plasma (PRP). PRP aggregation was simultaneously determined by measuring the maximum percent decrease in optical density (OD) and laser light scatter (LS) intensity using an aggregometer, PA-200 (Kowa). ADP 100 nmol/L was used as an agonist for platelet aggregation and added to PRP 60 seconds after the start of measurement. The principles of the LS method have been described previously. This method is based on the fact that the intensity of scattered light emitted from a particle increases in proportion to the square of its diameter. Particles with an intensity of 25 to 400 mV represented medium aggregates (25 to 50 square of its diameter). Particles with an intensity of 1000 to 2048 mV represented large aggregates (50 to 70 square of its diameter. Small aggregates usually contain approximately 70 to 1400 platelets. Generally, aggregates smaller than 10 m\(\mu\)m are found in the early phase of aggregation. Quantitative estimation was performed by determining the area under the curve from the sum of 30 measurements of the LS intensity. Data arising from this analysis cannot detect the any aggregation in response to 10 nmol/L ADP in normal or patient-derived platelets.

Other Assays

Cellular cholesterol was measured using a highly sensitive fluorometric method, as described previously. Protein was determined by the method of Lowry et al.

Results

Presence of hSRB1/CLA-1 Transcript in the Megakaryocytic Cell Line and Human Platelets

Previous studies showed that CD36 is found on the surface of platelets, and its presence affects platelet function. CD36 belongs to a family of proteins including hSR-B1/CLA-1. Whether hSR-B1/CLA-1 is present on platelets is not known. To answer this question, we probed for expression of hSR-B1/CLA-1 in human platelets and the megakaryocytic cell line, Mo7e. RT-PCR was used to probe for hSR-B1/CLA-1 mRNA in total RNA extracted from human platelets, Mo7e, and the positive control Hep G2 cells. The results revealed a single RT-PCR product of the expected size of 930 bp (Figure 1A).

Next we used Western blot analysis to determine whether hSR-B1/CLA-1 is present in extract from cells of interest and control cells. Cell extract probed with the antibody directed against hSR-B1/CLA-1 revealed a single band in all cells examined. Extract from human platelets and Mo7e cells had a single band of approximately 83 kDa. The mass of this protein matched the expected MW of the hSR-B1/CLA-1 protein (Figure 1B). However, mock transfected HEK 293 cells also contained detectable hSR-B1/CLA-1 protein, but this signal was much lower than that in the same cells stably transfected with the hSR-B1/CLA-1 cDNA. As expected, the positive control Hep G2 cells had abundant expression of hSR-B1/CLA-1. These findings indicate that both hSR-B1/CLA-1 mRNA and protein are present in human platelets and Mo7e cells.

HSR-B1/CLA-1 Is Present in Megakaryocytes

The distribution of hSR-B1/CLA-1 protein in adult human bone marrow cells was determined by immunostaining. Results arising from use of the hSR-B1/CLA-1 antibody showed that immunoreactivity was highest in megakaryocytes (Figures 2A and 2B). That this finding was specific is supported by lack of signal in megakaryocytes after use of nonspecific IgG to stain the cells (Figures 2C and 2D).
Additionally, all other hematopoietic cells in the marrow showed some reactivity to the hSR-B1/CLA-1 antibody. Together, these data suggest that expression of hSR-B1/CLA-1 is highest in megakaryocytes, with low levels evident in other cells of the bone marrow.

Expression of hSR-B1/CLA-1 Protein on the Surface of Human Platelets

The antibody against hSR-B1/CLA-1 was used to examine whether hSR-B1/CLA-1 protein could be detected on the surface of the platelets. For these studies, we used multiparameter flow cytometric analysis. Freshly isolated platelets were doubly labeled with a PE-anti-CD41 monoclonal antibody and FITC-anti-guinea pig IgG monoclonal antibody to detect anti-hSR-B1/CLA-1 antibody. Results showed that hSR-B1/CLA-1 was expressed in approximately 30% of CD41-positive platelets (Figure 3).

Next we probed for differential levels of hSR-B1/CLA-1 on platelets from patients with atherosclerotic disease compared with controls. A representative profile of hSR-B1/CLA-1 surface expression on the platelets from a healthy individual was compared with that of a patient with atherosclerotic disease (Figure 4A). The presence of hSR-B1/CLA-1 on the surface of platelets from the patient was clearly decreased compared with that of a subject without disease. This finding was confirmed using Western blot analysis. Results in Figure 4B show that abundance of hSR-B1/CLA-1 protein from the patient was decreased compared with that in a control patient. Together, these studies show that hSR-B1/CLA-1 expression in platelets is decreased in patients with atherosclerotic disease compared with the control group (Figure 5).

Abundance of hSR-B1/CLA-1 Correlates With Cholesterol Ester Content and Platelet Aggregation

The preceding results show the presence of hSR-B1/CLA-1 in human platelets, but the functional significance of this observation is not known. Therefore, we asked whether the presence of hSR-B1/CLA-1 correlated with its function in cholesterol metabolism and platelet aggregation. Platelets were isolated from healthy volunteers and patients with atherosclerotic disease. The abundance of hSR-B1/CLA-1 protein was correlated with plasma levels of HDL (Figure 6). Next, we measured the content of cholesterol ester in platelets using an ultrasensitive method, as described previously.22 Results showed that abundance of hSR-B1/CLA-1 on the surface of the platelets correlated negatively with accumulation of cholesterol ester in the platelets (Figure 6). In patients with atherosclerotic disease, the content of cholesterol ester in platelet cholesterol ester/platelet total cholesterol (PCE/PTC) (control, 16.1±1.1%; patient, 7.9±1.5%; mean±SE; P<0.0001) and abundance of hSR-B1/CLA-1 on the surface of the platelets (Figure 5) was significantly increased and decreased, respectively.

Whether differences of hSR-B1/CLA-1 on the surface of the platelets correlated with function was tested using laser LS, a measure of platelet aggregation. A representative profile of platelet aggregation using LS appears in Figure 4C. Platelet aggregation over time leads to a change in both OD and laser light scattering intensity. This technique enables the detection of small, medium, and large aggregates of platelets. In the absence of ADP, aggregation of the platelets from both patients with atherosclerotic disease and controls was the same. In contrast, formation of small aggregates after stimulation with 100 nmol/L ADP was significantly higher in patients with atherosclerotic disease compared with the controls (P<0.05). Another way to view this finding is that aggregation of platelets with low levels of hSR-B1/CLA-1 was high, thus underlining the inverse relationship between hSR-B1/CLA-1 expression and function of the platelet.
ever, accumulation of cholesterol ester and platelet aggregation did not significantly correlate with plasma cholesterol, HDL, LDL, or triglycerides (data not shown). These results suggest that abundance of hSR-B1/CLA-1 protein correlates inversely with both cholesterol ester content and ADP-stimulated aggregation of the platelets, two parameters that reflect function of the platelets.

Discussion

In this study, we examined the expression of hSR-B1/CLA-1 in human platelets and megakaryocytes. The results of our studies show that platelets and its precursor, the megakaryocyte, expressed hSR-B1/CLA-1 mRNA and the protein. Abundance of the hSR-B1/CLA-1 protein on human platelets correlated with serum levels of HDL (Figure 6). In contrast, hSR-B1/CLA-1 expression was negatively correlated with cholesterol ester content in the platelets (Figure 6). The significance of this inverse relationship was evident in patients with atherosclerotic disease. In these patients, de-
creased expression of hSR-B1/CLA-1 correlated inversely with the content of cholesterol ester in platelets and their ability to aggregate. These two parameters were significantly increased (Figure 6) in platelets with low levels of hSR-B1/CLA-1. Together, the findings reported here suggest an important relationship between hSR-B1/CLA-1 expression and the function of human platelets. The possibility that a deficiency in the platelet levels of CLA-1 may arise from a generalized decrease in the levels of this protein cannot be excluded at this point. Many factors, including hormones (ACTH and estrogen), lipids (cholesterol, oxidized LDL, and polyunsaturated fatty acids), and other agents (PPAR agonist and vitamin E) modulate the expression of hSR-B1/CLA-1. These findings limit our ability to make general comments, because a few study patients were treated with medications that may affect expression. Expanded clinical studies will be needed to clarify the role of hSR-B1/CLA-1 in platelets.

How do we relate the changes in hSR-B1/CLA-1 expression with cholesterol metabolism? The present belief is that the protective effect of HDL comes from its participation in RCT. This is a normal physiological process whereby cholesterol from cells in the arterial wall may be transported on HDL particles and shuttled to the liver for additional metabolism and disposal. Efflux of cellular free cholesterol from peripheral cells to the acceptor HDL particles is the first step of this process. Several reports have suggested that rodent SR-B1, a protein that is functionally related to hSR-B1/CLA-1, is an attractive candidate receptor that selectively takes up HDL cholesterol ester. SR-B1 is believed to play an important role as a docking receptor for HDL in connection with selective uptake of cholesterol esters. It is tempting to speculate that decreased levels of hSR-B1/CLA-1 in platelets, isolated from patients with atherosclerotic disease, reflect a decrease in RCT and thus enhance their risk for developing the disease.

The cloning of hSR-B1/CLA-1 was facilitated by amino acid sequence homologies that were highly conserved among CD36 and LIMPII. CD36 is present in megakaryocytes, monocytes, capillary endothelium, and also platelets. The interaction of CD36 with the fibrinogen-liganded form of GP IIb/IIIa is postulated to stabilize platelet aggregation. This hypothesis is supported by data showing that in SR-B1/apolipoprotein E double-knockout mice, SR-B1 can protect against the erosclerotic effect of SR-B1 expression in apolipoprotein E knockout mice suggests that pharmacologic stimulation of endogenous SR-B1 activity may be antiatherogenic, possibly related to its role in RCT. The antiatherosclerotic effect of SR-B1 expression in apolipoprotein E knockout mice suggests that pharmacologic stimulation of endogenous SR-B1 activity may be antiatherogenic, possibly related to its role in RCT. The antiatherosclerotic effect of SR-B1 expression in apolipoprotein E knockout mice suggests that pharmacologic stimulation of endogenous SR-B1 activity may be antiatherogenic, possibly related to its role in RCT. The antiatherosclerotic effect of SR-B1 expression in apolipoprotein E knockout mice suggests that pharmacologic stimulation of endogenous SR-B1 activity may be antiatherogenic, possibly related to its role in RCT.
In summary, the expression levels of hSR-B1/CLA-1 correlate positively with plasma HDL but negatively with cholesterol content in and aggregation of platelets. These findings raise the possibility that a measurement of the hSR-B1/CLA-1 expression on human platelets may provide a valuable insight that reflects the status of RCT in patients with atherosclerosis.

References

Expression of Human Scavenger Receptor B1 on and in Human Platelets
Hitomi Imachi, Koji Murao, Wenming Cao, Satoshi Tada, Tomohiko Taminato, Norman C.W. Wong, Jiro Takahara and Toshihiko Ishida

Arterioscler Thromb Vasc Biol. 2003;23:898-904; originally published online March 20, 2003; doi: 10.1161/01.ATV.0000067429.46333.7B
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/23/5/898

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/