Purification and Measurement of HDL3-binding Proteins in Human Peripheral Blood Mononuclear Cells

Hiroya Hidaka,1 Minoru Tozuka,2 Kazuyoshi Yamauchi,2 Hiroyoshi Ohta,1 Jun Nakayama,3 and Tsutomu Katsuyama2
1 Shinshu University School of Health Sciences, Matsumoto, Nagano, Japan
2 Department of Laboratory Medicine, Shinshu University Hospital and School of Medicine
3 Department of Pathology, Shinshu University School of Medicine

Abstract. We previously identified a binding site for high density lipoprotein-3 (HDL3) on the surface of human peripheral blood monocytes. Here we describe the purification and measurement of HDL-binding proteins present on these cells. Purification of HDL-binding proteins was achieved by chromatography using DEAE ion-exchange, wheat germ lectin, and apoHDL3 affinity columns. Subsequent use of SDS-PAGE and ligand blotting lead to the identification of two major proteins with apparent molecular masses of 100 and 120 kDa, plus several smaller proteins that appeared to be degradation products. Analysis of purified HDL-binding proteins by reverse-phase HPLC showed that the two main proteins at 100 and 120kDa were eluted in 65 and 70% acetonitrile, respectively, indicating the proteins are strongly hydrophobic.

To measure the amount of HDL-binding protein present in CHAPS-solubilized human mononuclear cells, a 96-well plate assay was developed that was based on the same principles as the purification method. The present study of HDL-binding proteins in mononuclear cells advances our understanding of the physiological roles and fate of HDL.

Keywords: HDL3, HDL-binding protein, peripheral blood mononuclear cells, RP-HPLC

Introduction

Plasma levels of high-density lipoprotein (HDL) are inversely correlated with the risk of atherosclerotic coronary artery disease [1]. HDL is believed to play a role in the process of reverse cholesterol transport (RCT) [2]. Although the interaction of HDL with binding sites on the surface of peripheral blood cells appears to be an important first step in RCT [3-5], the nature of this association is not fully understood.

Several laboratories have reported the presence of HDL-binding proteins on the plasma membranes of various cell types, including fibroblasts, hepatocytes, adipocytes, and macrophages [6-14]. Binding to these proteins may promote the efflux of free cholesterol from the cell, and may also promote cholesterol uptake into the cell membrane [15]. The involvement of specific HDL receptors has been confirmed by the identification of SR-B1 and CLA-1, which both mediate cholesterol transfer to cells [16-18].

We previously identified specific HDL binding sites on human blood monocytes using flow cytometric analysis and fluorescence-labeled HDL3 [19]. Partial purification of HDL binding proteins from these cells was achieved by DEAE ion-exchange chromatography. HDL3 ligand blotting assays showed that these monocyte HDL-binding proteins had binding parameters, specificity, and molecular weights similar to HB1 and HB2 from rat and human liver plasma membranes [19].

Abbreviations: HDL, high density lipoprotein; PO, peroxidase; WG, wheat germ lectin; PBS, phosphate-buffered saline (10 mM Na2HPO4/NaH2PO4 (pH 7.4), 150 mM NaCl); CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; Trizma-base, tris(hydroxymethyl)-aminomethane; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TMB, tetramethylbenzign dihydrochloride; Tween 20, polyoxyethylene sorbitan monolaurate.
This report describes the purification of HDL binding proteins from human blood mononuclear cells, as well as a microplate assay to measure these proteins in solubilized cells. This characterization and measurement of HDL-binding proteins advances our knowledge of the processes that influence plasma HDL levels.

Materials and Methods

**Chemicals.** NaCl, NaBr, peroxidase labeled-wheat germ lectin (POD-WG), and trifluoroacetic acid (TFA) were from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Lymphoprep and goat anti-human-apo A-I antibody (70 mg protein/ml) were from Dai-ichi Pure Chemical Co., Tokyo, Japan. Phosphate-buffered saline and peroxidase-conjugated anti-goat IgG (rabbit) were from Medical and Biological Laboratories Co., Tokyo, Japan. Heparin Sepharose CL6B, wheat germ lectin Sepharose 6MB (WG-Sepharose), CNBr-Sepharose, DEAE-Sephadex gels, and RESOUSE-RP column were from Pharmacia Biotech, Uppsala, Sweden. Protein assay kit containing bicinchoninic acid (BCA) was from Pierce Chemical Co., Rockford, IL. Centricon filters were from Amicon, Danvers, MA. Gradient gels (8-16%) containing SDS were from TEFCO Co., Tokyo, Japan. CHAPS, Trizma-base, and PMSF were from Sigma, St. Louis, MO. Chromogen TMB solution and buffer/substrate TMB (containing urea and hydrogen peroxide) were from Dade-Behring, Marburg, Germany. Tween 20 and molecular mass standards for SDS-PAGE were from Bio-Rad Laboratories, Hercules, CA.

**Preparation of lipoproteins.** Human HDL_{3} (d, 1.125-1.21 g/ml) was isolated from fresh human plasma by ultracentrifugation according to Havel et al [20]. HDL_{3} equilibrated by dialysis against 50 mM Tris-HCl buffer (pH 7.4, containing 0.05 M NaCl) was applied to a heparin-sepharose CL6B column and eluted with the same buffer, to obtain HDL_{3}-without-apoE. This HDL_{3} was concentrated by centrifugation through Centricon membrane filters and then characterized by SDS-PAGE using the Pharmacia Fast-System (Pharmacia Biotech). The protein concentration of lipoproteins was assayed with the BCA protein assay kit.

**Preparation of mononuclear cells.** Human blood mononuclear cells were prepared by a modified method of Ting et al [21]. Fresh human blood (5 ml), collected in tubes containing heparin or EDTA, was diluted 2-x with PBS, then layered gently onto 2 ml of Lymphoprep (d, 1.077 g/ml). After centrifugation (400 x g, 30 min, 20°C), the mononuclear cells were collected from the intermediate phase, washed twice with PBS at 4°C, and resuspended in 1 ml of PBS. Mononuclear cells were counted with a blood cell counter (Sysmex NE-7000, Toairyoudenshi, Tokyo, Japan).

**Purification of HDL binding proteins.** Human mononuclear cells were solubilized with Tris/HCl buffer (50 mM, pH 8.0) containing 20 mM CHAPS and 1 mM PMSF for 1 hr at 4°C [6]. The CHAPS-solubilized mononuclear cells were loaded onto a DEAE-Sephalac column equilibrated with Tris/HCl buffer (50 mM, pH 7.4, containing 0.15 M NaCl, 10 mM CHAPS and 1 mM PMSF), and the HDL-binding protein-rich fraction was eluted with the same buffer containing 0.2 M NaCl. This fraction was loaded onto a wheat germ lectin-Sepharose 6MB column equilibrated with Tris/HCl buffer (50 mM, pH 7.4, containing 0.2 M N-acetyl-D-glucosamine. This fraction was adjusted to 6 mM CHAPS and 20% glycerol and loaded onto an apo-HDL (mainly apoA-I) affinity chromatography column equilibrated with Tris/HCl buffer (50 mM, pH 7.4, containing 0.1 M NaCl, 6 mM CHAPS and 20% glycerol) and the HDL-binding proteins were eluted with 0.2 M NH_{4}OH (approximately pH 10.5) containing 10 mM CHAPS. The elution fraction was immediately adjusted to neutral pH with 0.5 M Tris/HCl buffer (pH 7.4, containing 10 mM CHAPS). HDL-binding proteins were concentrated using an Amicon ultrafiltration cell, and equilibrated with Tris/HCl buffer (50 mM, pH 7.4, containing 0.1 M NaCl and 10 mM CHAPS).
Preparative electrophoresis was performed using 4-12% SDS-PAGE, according to Laemmli [22]. After electrophoresis, proteins in the gel were stained with Coomassie brilliant blue R-250 (0.1% in 40% methanol/10% acetic acid) for 10 min. The gel was quickly destained in 40% methanol/10% acetic acid for 20 min. The gel was placed in water for 30 min to remove the methanol and acetic acid. The stained proteins were extracted from the gel by diffusional elution in 50 mM NH₄HCO₃ containing 1% SDS, and then precipitated with chloroform/methanol (1:3, v/v) as described by Wessel and Flugge [23].

RP-HPLC. The HDL-binding protein fraction from apo-HDL affinity chromatography was adjusted to acid pH with 0.1% TFA (final concentration) and loaded onto an RP-HPLC column (RESOURSE-RP, 0.6 x 3 cm, particle size 15 µm) equilibrated with 0.1% TFA. After washing out the unbound fraction in 0.1% TFA, the bound fraction was eluted with a 0-100% gradient of acetonitrile solution containing 0.1% TFA. Absorbance of proteins was measured at 280 nm by a single path monitor (UV-1, Pharmacia Biotech, Uppsala, Sweden).

Measurement of HDL-binding proteins. Human mononuclear cells were solubilized with Tris/HCl buffer (50 mM, pH 8.0) containing 20 mM CHAPS and 1 mM PMSF for 1 hr at 4°C. Apo-HDL (50 µl of indicated concentration) was added to a 96-well plate (Nunc-immuno module, F8 MAXISORP type; Nunc, Roskilde, Denmark) and the plate was incubated at 37°C overnight. After washing with phosphate buffer containing 0.05% Tween, CHAPS-solubilized mononuclear cells were added, and the plate was incubated for 1 hr at room temperature. The plate was washed and peroxidase-conjugated WG lectin (2 mg, containing 50% glycerol and 10 mM phosphate buffer with saline, pH 7.2) was added, and incubated for 1 hr at 37°C. After washing with PBS, HDL-binding proteins were detected by addition of chromogen-TMB-solution and spectrophotometry at 450 nm and 650 nm (ie, the blank wavelength).

Ligand blotting and protein staining. Sample buffer was added to protein samples to give a final concentration of 0.1 M Tris/HCl, pH 6.8, 2% glycerol, 0.1% bromophenol blue and 1% SDS. Samples were applied to SDS-containing 8-16% polyacrylamide gels, and electrophoresed using the TEFKO-system (TEFCO, Tokyo, Japan) using the manufacturer’s instructions, and then electroblotted onto nitrocellulose membrane (Toyo Roshi, Tokyo, Japan). Ligand blotting was performed as previously described [19]. Nitrocellulose-bound proteins were visualized by gold staining.

Results

Purification of HDL-binding protein. The SDS-PAGE data in Fig. 1 show the results at various stages of purification of HDL-binding proteins from human mononuclear cells.

Fig. 1. Purification of HDL-binding proteins from human mononuclear cells. Fractions from each chromatography stage were electrophoresed on 8-16% SDS-PAGE gels and blotted onto a nitrocellulose membrane, which was gold stained. Lane 1, molecular mass markers; lane 2, CHAPS-solubilized extract of mononuclear cells; lane 3, DEAE-Sepahel fractions (0.05 - 0.2M NaCl); lane 4, WG lectin-bound fractions; lane 5, apo-HDL affinity gel bound fraction.
human blood mononuclear cells. CHAPS-
solubilized mononuclear cells (Fig. 1, lane 2) were
chromatographed on a DEAE ion-exchange column
and HDL-binding proteins eluted with 0.2 M NaCl
(Fig. 1, lane 3). This fraction was loaded onto a WG
lectin column and HDL-binding proteins eluted
using 0.2 M N-acetyl-D-glucosamine (Fig. 1, lane
4). This eluted fraction was applied to an apo-HDL
column, and the HDL-binding proteins eluted using
0.2 M NH$_4$OH (approximately pH 10.5) (Fig. 1,
lane 5). Two major HDL-binding proteins were
identified with apparent molecular weights of 100
and 120 kDa.

Characterization of HDL-binding proteins. The two
major proteins eluted from the apo-HDL affinity
column were tested for HDL binding activity. The
proteins were purified by 2 approaches. In the first
approach, the eluted proteins were subjected to
preparative SDS-PAGE, stained, eluted from the gel,
and electrophoresed using analytical SDS-PAGE,
after which the proteins were electroblotted and then
either ligand blotted using HDL as a ligand (Fig. 2,
A) or gold stained (Fig. 2, B). The data in Fig. 2

Fig. 2. Ligand blotting and gold staining of the preparative SDS-PAGE fractions. HDL-binding proteins purified by apo-HDL
affinity chromatography were subjected to preparative SDS-PAGE and stained with Coomassie Brilliant Blue R250. The proteins
were liberated by diffusional elution in 50 mM NH$_4$HCO$_3$ containing 1% SDS and subjected to analytical SDS-PAGE, and
analyzed by ligand blotting (panel A) and protein staining (panel B).

Fig. 3. RP-HPLC of HDL-binding proteins. The HDL-binding protein-rich fraction from the apo-HDL affinity
column was adjusted to acid pH (final concentration of 0.1%
TFA) and loaded on an RP-HPLC column. After washing the
column with 0.1% TFA, the bound proteins were eluted using
a 0-100% acetonitrile gradient in 0.1% TFA, and eluted
fractions (1-7) were collected. The absorbance of proteins was
measured at 280 nm.
show that 2 predominant HDL-binding proteins were recovered from preparative SDS-PAGE (fractions 3 and 5), as well as some lower molecular weight proteins (fractions 4, 6, 7, and 8). The lower bands may be degraded proteins from the 100 and 120 kDa proteins. Lane 9 shows the molecular mass calibration marker.

In the second approach, HDL-binding proteins isolated by apo-HDL affinity chromatography were adjusted to acid pH by TFA (0.1% final concentration), and then loaded onto RP-HPLC column. A concentration gradient of acetonitrile was used to elute proteins. Two major peaks eluted at apparent 65 and 70% acetonitrile respectively (Fig. 3). These two protein peaks were subjected to SDS-PAGE, electroblotted onto a nitrocellulose membrane and then gold stained. Lanes 1-7 correspond to fraction numbers 1-7 in Fig. 3; lane 8 contains the apo-HDL affinity bound fraction and lane 9 contains the molecular mass markers.

First, we examined the effect on binding of increasing the concentration of coating apo-HDL. Using a range of 0-7.5 µg apoHDL/well, we found that constant binding was achieved at a concentration of approximately 5 µg/well (Fig. 5, A). Based on these observations, subsequent studies all used a coating concentration of 5 µg/well. The binding of HDL-binding proteins to adsorbed apo-HDL was almost completely inhibited by pre-incubation with an anti-apo-AI antibody (Fig. 5, B). For assay calibration, the HDL-binding protein purified by apo-HDL affinity chromatography was used as a standard (Fig. 5, C). Protein concentration of 1 µg/ml in the apo-HDL affinity chromatography bound fraction was considered to represent 1 unit of HDL-binding proteins. Increasing the amount of HDL-binding proteins resulted in a linear increase in absorbance over a range of 0 to 1 µg/ml. Concentration-dependent binding studies were performed using various dilutions of CHAPS-solubilized mononuclear cells (Fig. 5, D). A linear increase in absorbance was observed over the range of 0-20 µg/ml of solubilized mononuclear cells.

**Discussion**

This report describes the purification and characterization of HDL-binding proteins from human blood mononuclear cells, and the development of an assay for these proteins. The two HDL-binding proteins identified possess specific binding activity to apo-HDL, and have apparent molecular weights of 100 and 120 kDa. The assay system successfully measured HDL-binding proteins in CHAPS-solubilized mononuclear cells.

HDL-binding proteins were purified using WG lectin affinity chromatography, indicating that they
are glycoproteins. The HDL-binding proteins appear strongly hydrophobic. Although human blood mononuclear cells comprise mainly monocytes and lymphocytes, it seems likely that the HDL-binding proteins identified in this study were derived from monocytes, since previous flow cytometry experiments using FITC-labeled ligands identified HDL-binding sites on monocytes but not lymphocytes [19].

The data indicate that HDL-binding proteins are hydrophobic polypeptides with glycosides, which suggests they are membrane proteins. It was recently reported that SR-B1 and CLA-1 are specific HDL receptors [16-18]. However, there is little evidence that the HDL-binding proteins reported here corresponded to SR-B1 and CLA-1. SR-B1 and CLA-1 show affinity for LDL in addition to HDL [24], but the HDL-binding protein from human monocytes shows specific affinity with HDL [19]. The molecular weights of CLA-1 (Mr 83 kDa) and SRBI (Mr 80 kDa) are different from the HDL-binding proteins, 120 and 100 kDa [19].
In the present study, 2 major HDL-binding proteins were identified, as well as a number of smaller proteins with binding activity. It is possible that these smaller proteins are degradation products of the two main proteins, and that degradation occurred during NH₄CO₃ elution from preparative SDS-PAGE. Loss of sugar chains from the 2 major proteins may explain our observations, since N-glycanase treatment reduced the molecular weight of HDL-binding proteins derived from rat liver plasma membranes, and these smaller proteins also maintained binding activity [6].

It is unclear whether the two HDL-binding proteins identified in the present study have similar primary structures to each other, or to the recently cloned and sequenced HB2 from rat liver plasma membrane [25]. It will be necessary to characterize both proteins further in order to determine their physiological functions.

We previously reported the identification and measurement of HDL-binding activity in human blood monocytes using flow-cytometry and a fluorescence-labeled ligand [19]. Although flow-cytometry assay enabled the identification and partial characterization of HDL-binding sites, it was difficult to estimate the quantity of protein. Here we presented details of an assay designed to overcome this limitation. The binding assay was developed based upon the purification assay, that is, association of HDL-binding proteins with apoA-I and WG lectin. The optimized assay appeared specific for HDL-binding proteins and was reproducible in the range of the standard curve. This new assay is likely to facilitate investigations of the factors that regulate HDL binding.

In conclusion, the present report describes the purification and measurement of HDL-binding proteins in mononuclear cells and provides new information that advances our understanding of the regulation of plasma HDL concentrations.

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References


