The identification of specific high density lipoprotein$_3$ binding sites on human blood monocytes using fluorescence-labeled ligand

Hiroya Hidaka,[1,*] Eiko Hidaka,[*] Minoru Tozuka,[*] Jun Nakayama,[*] Tsutomu Katsuyama,[*] and Noel Fidge[†]

Department of Laboratory Medicine,* Shinshu University School of Medicine, Matsumoto, Japan, and Lipoprotein and Atherosclerosis Unit,† Baker Medical Research Institute, Melbourne, Australia

Abstract **We previously reported the identity and purification of two HDL$_3$-binding proteins in rat liver plasma membranes. As these proteins are candidate high density lipoprotein (HDL) receptors and probably multifunctional, including a role in HDL metabolism, we have considerable interest in identifying corresponding proteins that are present in human tissue. This report describes the identification of HDL$_3$-binding sites on human monocytes with the use of fluorescence microscopy and flow cytometry assay. After the incubation of mononuclear cells from human blood with fluorescein isothiocyanate (FITC)-labeled human HDL$_3$, fluorescence micrographs showed dense signals of fluorescent grains on monocytes, but not lymphocytes. A significant increase in FITC intensity on monocytes, but not lymphocytes, was observed by flow cytometry analysis, and the interaction between FITC-HDL$_3$ and human monocytes was concentration-dependent. Although very low density (VLDL) and low density lipoprotein (LDL) were ineffective competitors and HDL$_2$ only partially competed for binding, a 50-fold concentration of HDL$_3$ did compete effectively for binding of FITC-HDL$_3$ to human monocytes. Trypsin treatment reduced the FITC intensity of monocytes, showing that a portion of cell-associated FITC-HDL$_3$ remained bound to the cell surface. Two major HDL-binding proteins were identified in CHAPS-solubilized human mononuclear cells by ligand blotting, using HDL$_3$ as the ligand. Both showed similar binding parameters, specificity, and molecular weight identical to HB$_1$ and HB$_2$ from rat liver plasma membrane. We conclude that corresponding candidate HDL receptors or a similar receptor complex also exist on human blood monocytes.**—Hidaka, H., E. Hidaka, M. Tozuka, J. Nakayama, T. Katsuyama, and N. Fidge. **Identification of specific high density lipoprotein$_3$ binding sites on human blood monocytes using fluorescence-labeled ligand.** ***J. Lipid Res.*** **1999.** 40: **1131–1139.**

Supplementary key words HDL$_3$ • HDL-binding protein • monocyte • flow cytometry • fluorescent isothiocyanate

High density lipoprotein (HDL) plays a major role in reverse cholesterol transport from peripheral tissues to the liver. The mechanisms involved in HDL- mediated cholesterol efflux from peripheral cells and the delivery of sterol to the liver are not fully understood, but the involvement of specific HDL receptors in the process has recently been confirmed by the identification of SR-B1 and CLA-1 which mediates both cholesteryl ester transfer to some cells (1–3) and cholesterol efflux from others (4). Other high-affinity HDL-binding sites have been detected in a variety of tissues and cells including rat, porcine, and human liver plasma membrane (5–9), cultured mouse adipocytes (10, 11), human skin fibroblasts (12, 13), rat testis (14), and macrophages (15), but their identity as functional HDL receptors is less secure.

Our group previously reported the identification of two HDL-binding proteins, HB$_1$ (120 kDa) and HB$_2$ (100 kDa), in rat and human liver plasma membrane by HDL$_3$-ligand blotting (5) and subsequently cloned HB$_2$ (16) which was shown to have significant homology with some adhesion molecules, particularly ALCAM (17). Transfection of cells with HB$_2$ was associated with increased binding of HDL$_3$ but not selective transfer of HDL cholesteryl ester to cells (16).

Macrophages derived from monocytes possess candidate HDL receptors that bind HDL or modified HDL (15, 18) indicative of a role for HDL in monocyte lipid metabolism, a plausible suggestion considering the exposure of monocytes to lipoproteins in plasma. This hypothesis has been strengthened by the recent observation that HB$_2$, barely detectable in THP-1 cells (a monocyte B cell line), is dramatically up-regulated after PMA-induced differentiation of those cells into macrophages and by the observa-

Abbreviations: HDL, high density lipoprotein; HB, HDL-binding; LDL, low density lipoprotein; FITC, fluorescein isothiocyanate; FH, familial hypercholesterolemia; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electorphoresis; BCA, bicinchoninic acid; VLDL, very low density lipoprotein.

[1] To whom correspondence should be addressed.

tion that HB_2 mRNA levels decline after cholesterol loading of those cells (16).

Flow cytometry is a technique for analyzing cells or particles according to their size and amount of fluorescence bound to them. Nagano et al. (19) recently used flow cytometry to measure low density lipoprotein (LDL)-receptor activity in human plasma lymphocytes and the data correlated well with LDL-receptor activity in fibroblasts of patients with familial hyperlipidemia (FH) and normal subjects assessed by a binding assay using ^{125}I-labeled LDL in vitro.

We have modified a novel fluorescent probe method (20) to investigate the interaction between HDL and monocytes and investigate putative HDL receptor activity in these cells. We identified specific binding sites for fluorescein isothiocyanate (FITC)-HDL_3 using fluorescence microscopy and a flow cytometry assay and evidence for HDL-binding proteins by ligand blotting in human blood monocytes.

MATERIALS AND METHODS

Chemicals and materials

FITC was purchased from Wako Chemical Co. (Osaka, Japan). Lymphoprep and anti-human-apoA-I (rabbit) were obtained from Daichi Pure Chemical Co. (Tokyo, Japan). Phosphate-buffered saline (PBS), peroxidase-conjugated anti-rabbit IgG (goat), phycoerythrin (PE)-labeled CD14, a specific antibody for monocytes, and FITC-labeled CD4, a specific antibody for lymphocytes, were obtained from Medical and Biological Laboratories Co. (Tokyo, Japan). PD-10 columns, heparin–Sepharose CL6B, 4–30% polyacrylamide gradient gels (PAA4/30) using the GE-2/4 system, and DEAE-Sephacel gels were obtained from Pharmacia Biotech (Uppsala, Sweden). Trypsin (1:250) was obtained from Difco Laboratories (Detroit, MI). The Simultest™ Control γ1/γ2a containing mouse IgG_1-FITC and IgG_2a-PE with gelatin and 0.1% azide was obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA). Bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce Chemical Co.(Rockford, IL). Centricon membrane filters were obtained from Amicon (Danvers, MA). Polyacrylamide gradient gels (8–16%) containing sodium dodecyl sulfate (SDS) were obtained from TEFCO Co. (Tokyo, Japan). CHAPS and TRIZMA base were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of human lipoproteins

Human very low density lipoprotein (VLDL) ($d < 1.006$ g/ml), low density lipoprotein (LDL) (d 1.006–1.063 g/ml), HDL_2 (d 1.063–1.125 g/ml), and HDL_3 (d 1.125–1.21 g/ml) were isolated from fresh human sera by ultracentrifugation according to the method of Havel, Eder, and Bragdon (21). 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. The other 1ipoproteins were desalted on PD-10 columns equilibrated with 50 mm Tris-HCl buffer (pH 7.4) containing 0.15 m NaCl, and concentrated by centrifugation through Centricon membrane filters and then characterized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Pharmacia Fast-system (Pharmacia Biotech). The protein concentration of lipoproteins was determined using the BCA protein assay reagent.

FITC-labeled HDL_3

FITC-labeled HDL_3 (FITC-HDL_3) was prepared using a modification of the method of Riggs et al. (20). FITC (1 mg) in 0.1 m carbonate buffer (pH 9.6) was added to 10 mg of HDL_3 protein previously adjusted to pH 9.6. The mixture was gently rocked for 1 h at room temperature, applied to a PD-10 column, and equilibrated with PBS for separating free FITC from conjugated-FITC-HDL_3. The conjugated-FITC was then equilibrated with PBS and concentrated using Centricon filters. The FITC-HDL_3 was divided into two portions and characterized by nondenaturing gradient gel PAGE and SDS-PAGE. One portion was mixed with glycerol and bromphenol blue, then applied in duplicate to wells of a native gradient gel. The other portion was mixed with 5% SDS containing glycerol and BPB and then applied in duplicate to wells of an SDS-PAGE gel.

Preparation of mononuclear cells from human blood

Human mononuclear cells were prepared according to a modified method of Ting and Morris (22). Fresh human blood (5 ml) collected in tubes containing heparin or ethylenediaminetetraacetic acid, dipotassium salt was diluted 2-fold with PBS, then layered gently onto 2 ml of Lymphoprep (d 1.077 g/ml). After centrifugation at 400 *g* for 30 min at 20°C, the mononuclear cells collected from the intermediate phase were washed twice with PBS at 4°C, and finally resuspended in 1 ml of PBS. Mononuclear cells were counted using a blood cell counter, Sysmex NE-7000 (Toa-iryoudenshi, Tokyo, Japan).

Fluorescence microscopy of mononuclear cells

For fluorescence microscopy, 1×10^5 mononuclear cells in 0.1 ml of PBS were incubated with 4 μg of FITC-HDL_3 with or without a 50-fold excess of unlabeled HDL_3, or with unconjugated FITC or FITC-CD4. Cells were then washed three times with 1.5 ml of PBS and suspended with 10 μl of 2.5% BSA. The cells that were suspended in PBS (approximately 1×10^5) were placed on glass slides using Cyto Spin (Sakuraseiki Co. Ltd. Nagano, Japan) in accordance with the manufacturer's instructions. Then the samples were stained with propidium iodide (400 ng/ml in antifade mounting solution, Vysis, IL) as counter staining, and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Fluorescence measurement

Fluorescence measurements were performed using a Shimadzu (Kyoto, Japan) RF-550 HPLC monitor spectrofluorometer. An aliquot of cells (250 μl) was added to a flow cell (12 μl) with the use of a peristaltic pump, and the fluorescence intensity at 520 nm was measured separately for the excitation wavelength of 490 nm.

Flow cytometry of mononuclear cells

A flow cytometry analysis of mononuclear cells was performed using the FACSort (Becton Dickinson, Sunnyvale, CA) in accordance with the manufacturer's instructions. In brief, mononuclear cells (approximately 1×10^5 in 100 μl of PBS) were incubated with 1 μg of FITC-HDL_3 protein for 1 h at 37°C. After washing with 50 vol of PBS, the cells were centrifuged at 3000 rpm for 3 min at 4°C, resuspended in 0.5 ml of PBS, and then analyzed by FACSort. In some experiments, mononuclear cells previously incubated with FITC-HDL_3 at 37°C for 1 h were treated with trypsin at 37°C for 15 min. The cells were then washed and resuspended in PBS and analyzed by the FACSort system. The peak channel of the logarithmic fluorescence histogram was used as a measurement of the amount of fluorescence bound to each particle. The cytometer electronically processes the electronic signals resulting from each cell, creating numeric values for the three parameters: forward scatter (FSC), side scatter

(SSC), and fluorescence 1 (FL1). It assigns each value a channel number, from 0 to 1023. These values are measurements of the relative light intensity scattered or emitted by the cell when it passes through the laser beam.

Partial purification of HDL-binding proteins from human mononuclear cells

The HDL-binding proteins from human monocytes were isolated and partially purified as described previously (3). Samples were solubilized with 30 mm CHAPS and 1 mm PMSF, and loaded onto a DEAE-Sephacel column equilibrated with 50 mm Tris-buffer (pH 8.0) containing 10 mm CHAPS (buffer A). After the column was washed with buffer A, HDL-binding proteins were eluted with Buffer A containing 0.2 m NaCl. The HDL-binding activities were detected by ligand blotting as previously described (2, 3).

RESULTS

Characterization of FITC-HDL_3

The particle size of FITC-HDL_3 and the composition of labeled-apoHDL_3 were characterized by gradient- and SDS-PAGE, respectively. The particle size of FITC-HDL_3 ranged from approximately 100 to 230 kDa as well as a native HDL_3, and FITC was associated with apoA-I and apoA-II, according to the SDS-PAGE (**Fig. 1**).

Fluorescence measurement

The association between HDL_3 and human mononuclear cells was assessed by saturation studies performed at 37°C for 2 h in PBS. Measurement of fluorescent intensity was calibrated using serially diluted fractions of FITC-HDL_3 in PBS; this demonstrated excellent linearity up to 600 ng/ml (**Fig. 2**, **A**). To estimate nonspecific binding of HDL_3, mononuclear cells were incubated with increasing concentrations of FITC-HDL_3 plus or minus 50-fold excess unlabeled HDL_3 at 37°C. Specific binding (total bind-

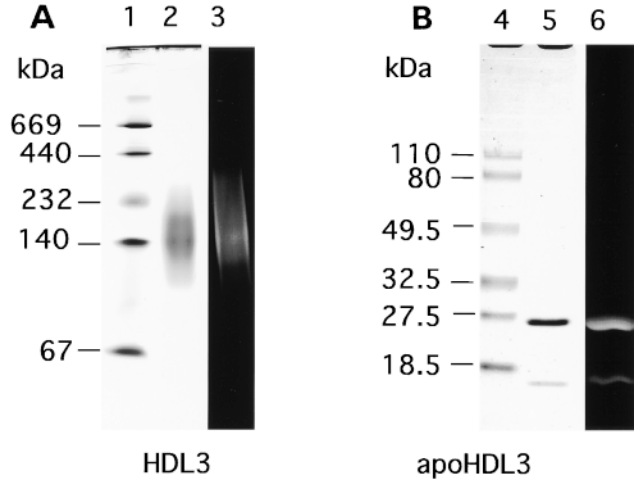

Fig. 1. Characterization of FITC-HDL_3. Human HDL_3 ($1.125 < d < 1.21$ g/ml) collected from fresh serum was adjusted to pH 9.6 with 0.1 m carbonate buffer, then incubated with FITC for 1 h at room temperature. The FITC-HDL_3 was separated from free FITC by PD-10 column chromatography in PBS, then characterized by nondenaturing polyacrylamide gel electrophoresis (native-PAGE, panel A) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, panel B). Bands on the native and SDS-PAGE were detected by staining with Coomassie Brilliant Blue R-250 (lanes 1, 2, 4, and 5) and using a fluorescence lamp (lanes 3 and 6). Lanes 1 and 4 show molecular mass calibration marker.

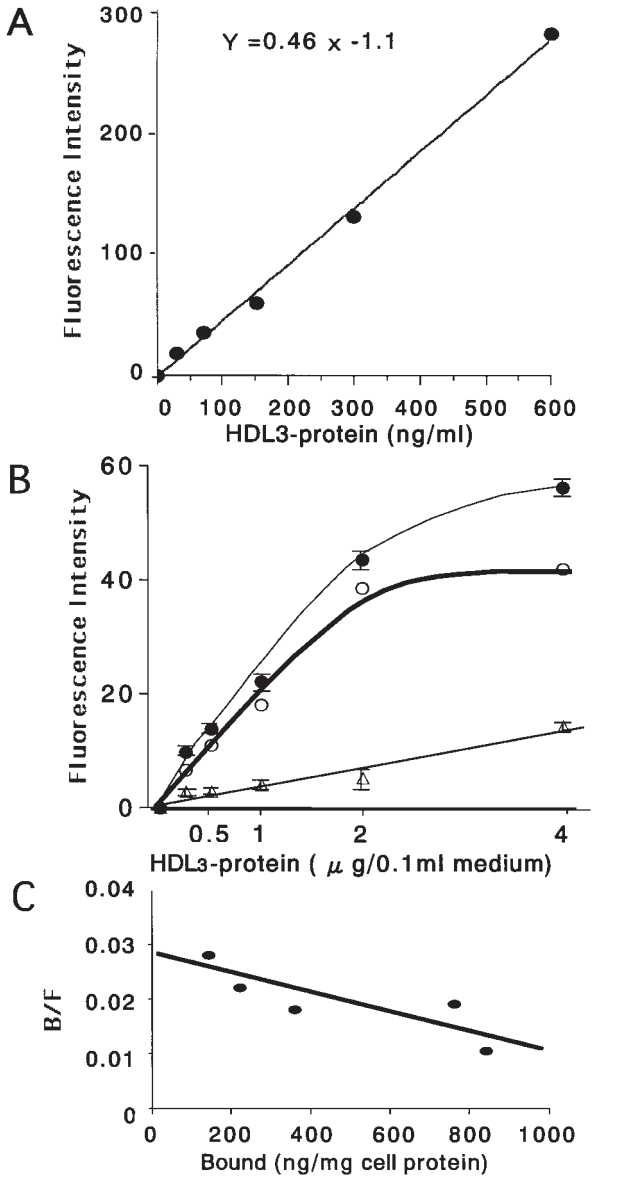

Fig. 2. Fluorescence measurement of concentration-dependent binding of FITC-HDL_3 to mononuclear cells. FITC-HDL_3 (600 ng/ml) was serially diluted with PBS and the fluorescent intensity was calibrated using a spectrofluorometer (panel A). The fluorescent intensity of mononuclear cells resuspended in PBS was also measured using a spectrofluorometer (panel B). Human mononuclear cells, collected from fresh blood as described in the Methods section, were incubated with increasing concentrations of FITC-HDL_3 plus (△) or minus (●) a 50-fold excess of unlabeled HDL_3. Specific binding (○) was calculated by subtraction of the values for binding in the presence of excess unlabeled HDL_3 (nonspecific binding, △) from the values for total binding (●). Values for specific binding were obtained from Scatchard plots (panel C). These data are the mean of triplicate points and are representative of two similar experiments each using one subject.

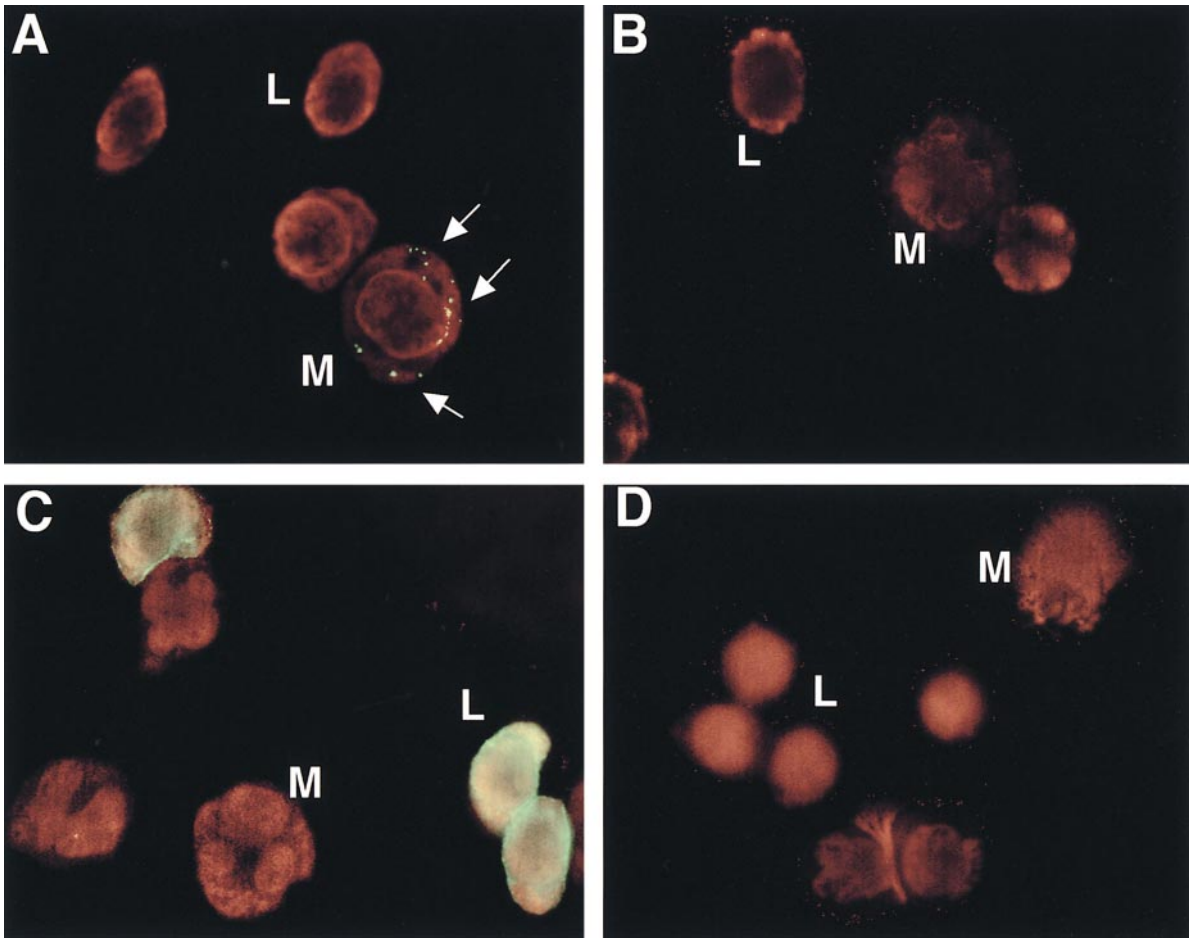

Fig. 3. Fluorescence microscopic identification of FITC-HDL_3-binding monocytes in mononuclear cells. Human mononuclear cells collected from blood as described in the Methods section were incubated with FITC-HDL_3 in the absence (panel A) or presence (panel B) of a 50-fold excess of unlabeled HDL_3, FITC-CD4 (panel C), or free FITC (panel D) for 1 h at 37°C. Smears of mononuclear cells were observed using fluorescence microscopy. The large-size cells are monocytes (M) and the smaller cells are lymphocytes (L). Fluorescent grains were visible only on monocytes incubated with FITC-HDL_3 (panel A), and fluorescent grains of FITC-CD4 were visible only on the surface of lymphocytes, not monocytes (panel C).

ing minus the binding in the presence of excess unlabeled HDL_3) showed saturation kinetics, with maximal binding achieved at approximately 4 μg of HDL_3 protein/0.1 ml of medium (Fig. 2, B). A Scatchard plot of the affinity binding was linear (Fig. 2, C), and a single binding site was seen with a K_d value of approximately 50 μg of HDL protein/ml.

Microscopic observation of FITC-HDL_3 binding to cells

Mononuclear cells were incubated with FITC-HDL_3, plus or minus 50-fold unlabeled HDL_3, FITC-CD4 (a cell surface marker of lymphocytes), and free FITC at 37°C for 1 h. Fluorescent grains were visible on monocytes but not lymphocytes after incubation with FITC-HDL_3 (**Fig. 3**), and were not present in cells incubated with a 50-fold excess of unlabeled HDL_3. FITC-CD4 bound only to lymphocytes, while free FITC did not bind to either cell type.

Flow cytometry of mononuclear cells

The flow cytometry procedure is also able to separate monocytes and lymphocytes amongst mononuclear cells. Mononuclear cells that were incubated with mouse IgG_1-FITC and IgG_{2a}-PE (Simultest Control γ1/γ2a) were analyzed using the FACSort system, which is shown in **Fig. 4**. The monocytes were identified as the larger cells from the forward scatter (FSC) light, properties dependent on cell size, and characterized by a stronger side scatter (SSC) light, an index of their greater internal cellular complexity compared to lymphocytes. Monocytes are circled (R1), and lymphocytes in the dense region in the area R2 (Fig. 4, A). Most of the monocytes, but not lymphocytes, bound the monocyte surface marker PE labeled-CD14 (Fig. 4, B). P1 is the fluorescence intensity of nonspecific binding or cells alone and P2 is the total bound fluorescence intensity.

After incubation of human mononuclear cells with FITC-HDL_3 at 37°C for 1 h in PBS, changes in the peak channel fluorescence intensity from monocytes and lymphocytes were measured by FLI, as shown in Fig. 4, C and D, respectively. The specific peak channel of the fluorescence intensity of the monocytes (M2, total binding, minus M1 which includes a 50-fold excess of unlabeled HDL_3)

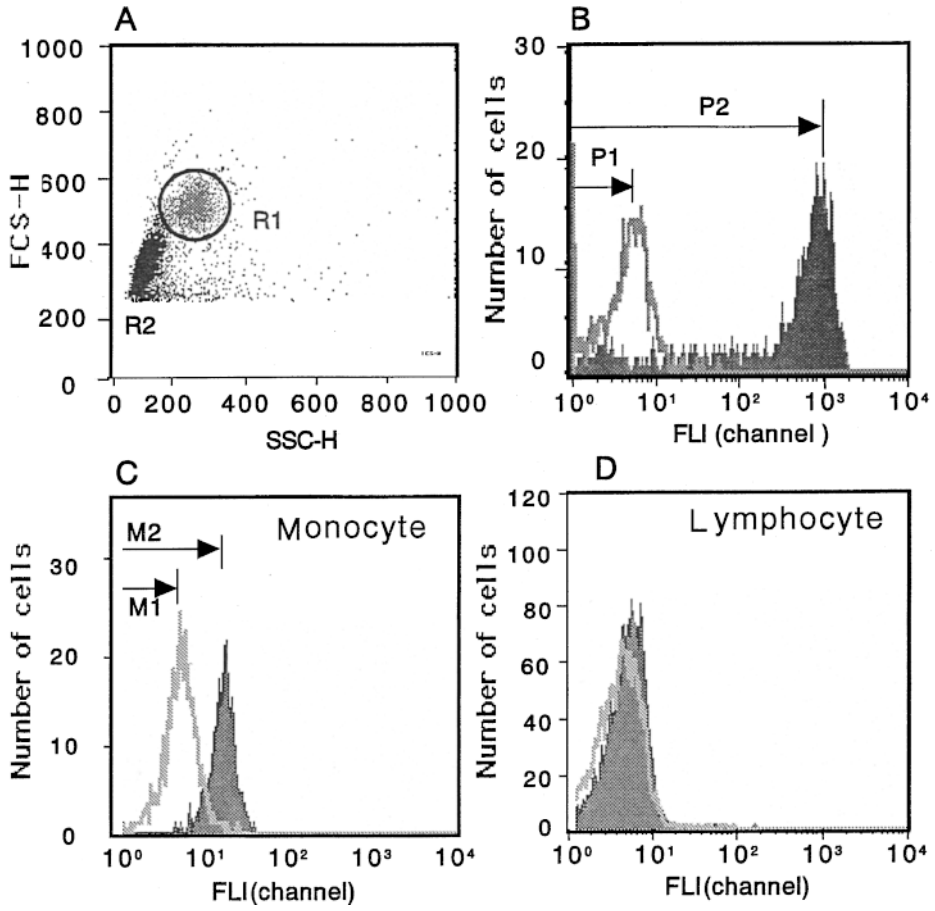

Fig. 4. Optimized FACSort results of mononuclear cells, FITC histogram of monocytes without and with PE-labeled CD14, and fluorescence flow cytometry analysis of monocytes and lymphocytes incubated with FITC-HDL_3, plus or minus 50-fold unlabeled HDL_3. Human mononuclear cells collected from fresh blood as described in the Methods section were incubated with Simultest™ Control $\gamma 1/\gamma 2a$ or monoclonal antibody against PE-labeled CD14 for 30 min at 4°C. After the cells were washed and resuspended with PBS, the mixture of cells was analyzed by FACSort. A dot density, two-parameter histogram illustrating the side light scatter height (SSC-H) on the x axis and forward light scatter height (FSC-H) on the y axis of human mononuclear cells is shown in panel A. Monocytes were gated with circle (R1) and lymphocytes with R2 in panel A. A one-parameter histogram illustrating the staining of monocytes with PE-labeled CD14 (P2, shadow peak) overlaid with a one-parameter histogram from controls (P1, solid line peak) is shown in panel B. The x axis is the fluorescence intensity (FLI, channel number), and the y axis is the number of events (cell count) in each channel. Human mononuclear cells (approximately 1×10^6/ml suspended in PBS) were incubated with 1 μg of FITC-HDL_3 (shadow peak) or FITC-HDL_3 plus a 50-fold excess of unlabeled HDL_3 (solid line peak) in 120 μl of incubation mixture for 1 h at 37°C. After the cells were washed and resuspended in PBS, the mixture of cells was analyzed by a FACSort system. One-parameter histograms illustrate the staining of monocytes (panel C) and lymphocytes (panel D) with FITC-labeled HDL_3 (M2, shadow peak) overlaid with one-parameter histograms from controls (M1, solid line peak). The x axis is the fluorescence intensity (FLI, channel number), and the y axis is the number of events (cells) in each channel.

was significantly increased by incubation with FITC-HDL_3 (Fig. 4, C), whereas the lymphocyte fluorescence was not changed (Fig. 4, D). Further, FITC-apoHDL_3, prepared by delipidation, bound to monocytes as did FITC-HDL_3. The correlation between FITC-apoHDL_3 (y-axis) and FITC-HDL3 (x-axis) binding obtained by the flow cytometry assay was $y = 0.4x + 5.5$ ($r = 0.747$, $n = 10$).

Binding studies with flow cytometry

Studies of the association of HDL_3 with human mononuclear cells were performed in a final reaction volume of 100 μl in PBS at 37°C. The association of 1 μg of FITC-HDL_3 with human mononuclear cells at 4° and 37°C in a time-course study is shown in **Fig. 5**, **A**. Temperature had a marked effect on HDL_3 binding to human monocytes. After 4 h at 37°C, the peak channel intensity (M2-M1) reached 35 channel, whereas at 4°C for 4 h the intensity remained unchanged. To further explore the importance of temperature, incubations were performed at 0°, 10°, 23°, and 37°C for 1 h which illustrated that FITC-HDL_3 binding increased in a linear fashion with rising temperature, as shown in Fig. 5, B.

Concentration-dependent binding studies were performed at 37°C for 1 h. The maximal binding of FITC-

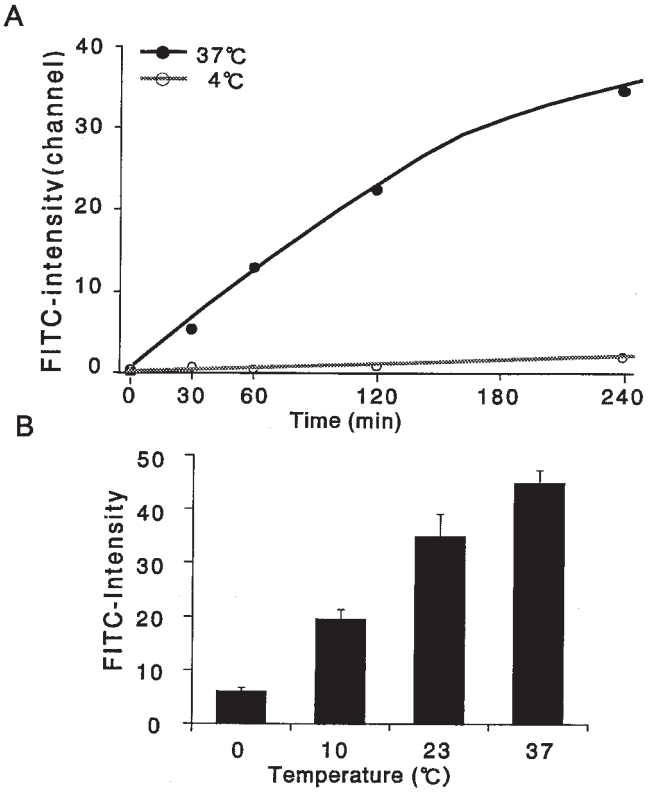

Fig. 5. Time course of FITC-HDL_3 binding to monocytes and temperature-dependent binding of FITC-HDL_3. Human mononuclear cells (approximately 1×10^6/ml suspended in PBS) were incubated with 1 μg of FITC-HDL_3 in 120 μl of incubation mixture for 0 to 240 min at 37°C or 4°C (panel A) and for 1 h at 4°C, 10°, 23°, or 37°C (panel B). After the cells were washed and resuspended in PBS, the FITC intensity (peak channel between control and treated cells, M2–M1) of the monocytes was measured by a FACSort, as described in Methods. These data are the mean of duplicate points and are representative of two similar experiments using one subject.

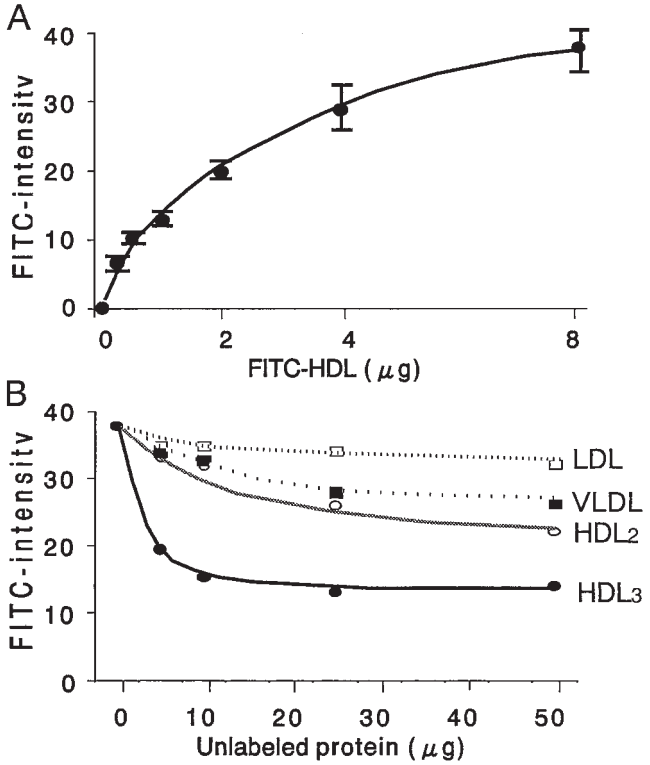

Fig. 6. Concentration-dependent binding of FITC-HDL_3 to human monocytes, and competition by unlabeled lipoproteins for the binding of FITC-HDL_3 to monocytes. Human mononuclear cells (approximately 1×10^6/ml suspended in PBS) were incubated with increasing concentrations (0 to 8 μg) of FITC-HDL_3 (panel A), and with 1 μg of FITC-HDL_3 and 5 to 50 μg of unlabeled lipoproteins in 100 μl of incubation mixture for 1 h at 37°C (panel B). After the cells were washed and resuspended in PBS, the FITC intensity (peak channel between control and treated cells, M2–M1) of the monocytes was measured by a FACSort, as described in Methods. These data are the mean of duplicate points and are representative of two similar experiments using one subject.

HDL_3 occurred between 4 to 8 μg of FITC-HDL_3 in 0.1 ml medium (**Fig. 6**, **A**). About 70% inhibition in binding was observed with a 50-fold excess of unlabeled HDL_3 (Fig. 6, B). VLDL and LDL competed minimally and HDL_2 was less effective than HDL_3, which produced a 50% displacement of FITC-HDL_3 at 50-fold excess concentration. To test whether these binding sites were sensitive to proteolytic cleavage, mononuclear cells were treated with trypsin. The peak channel of FITC intensity was decreased 40% by treatment with 0.2% trypsin for 1 h at 37°C (**Fig. 7**).

Detection and partial purification of HDL-binding proteins in mononuclear cells

Mononuclear cells solubilized with CHAPS were loaded onto a DEAE-Sephacel column. Fractions enriched with HDL-binding proteins were eluted with 0.2 m NaCl in the presence of 10 mm CHAPS. Binding activity was detected by ligand blotting as described in Methods. Two major HDL-binding proteins were identified with apparent M_r values of 120,000 and 100,000; a minor band (110 kDa) was also observed (**Fig. 8**).

DISCUSSION

The present results, obtained by fluorescence microscopy and a flow cytometry assay, demonstrated that human blood monocytes possess specific binding sites for FITC-labeled HDL_3.

We previously reported the presence of two HDL-binding proteins (HB_1 and HB_2) in rat and human liver plasma membranes (5, 6) and also showed that the expression of these candidate HDL-receptors could be reduced 50% by treating rats with the hyperlipidemic drug simvastatin (23). HB_2 levels could also be influenced by uploading macrophages with cholesterol (16), suggesting that this adhesion-type of HDL-binding protein may also be involved in cell lipid metabolism. Because at this stage of knowledge liver biopsies cannot be justified to assess these HDL receptor levels in relation to lipid perturbations, the aim of the present study was to investigate the existence of high-affinity HDL-binding sites in peripheral cells and the method described in this report appears to present an ideal solution to the problem. Monocytes are

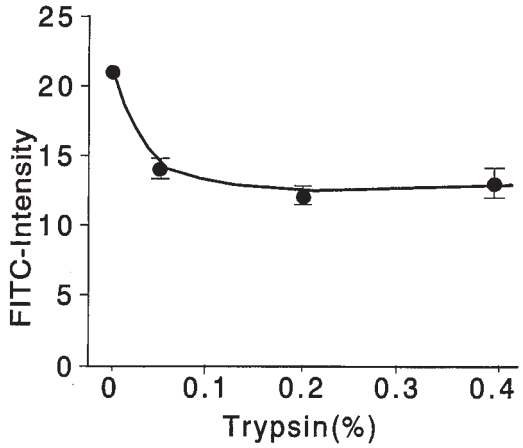

Fig. 7. Trypsin treatment of monocytes. Human mononuclear cells (approximately 1×10^6/ml suspended in PBS) were incubated with 1 μg of FITC-HDL_3 in 120 μl of incubation mixture for 1 h at 37°C. After the cells were washed and resuspended in PBS, cells were incubated with increasing concentrations of trypsin in PBS. Then, the FITC intensity (peak channel between control and treated cells) of the monocytes was measured by a FACSort, as described in Methods. These data are the means of duplicate data points obtained from one subject.

easily obtained from the blood of test subjects, and the combination of flow cytometry with fluorescence-labeled ligand (FITC-HDL_3) provides a simple, reproducible assay for comparing binding to candidate HDL receptors. Other work published previously (24) also suggests that the conjugation of HDL with the fluorescent marker FITC is a convenient and valid method for studying interactions between cells and HDL.

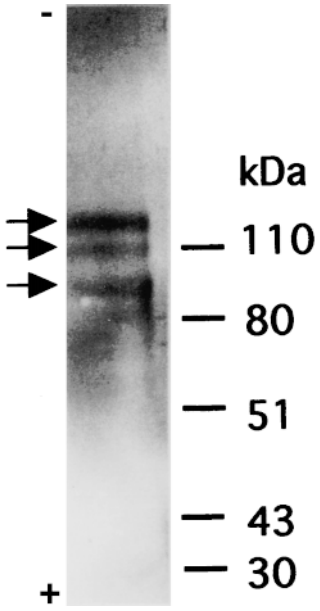

Fig. 8. Ligand blotting of monocyte HDL-binding proteins. CHAPS-solubilized human mononuclear cells were applied to a DEAE-Sephacel column equilibrated with buffer A (see text) and then washed with the same buffer. The HDL-binding protein-rich fractions were eluted with buffer A containing 0.2 m NaCl. HDL-binding proteins were detected by ligand blotting as described in Methods. The M_r standard markers are from Bio-Rad (Richmond, CA).

When FITC-HDL_3 was incubated with human monocytes at 37°C, a significant increase in fluorescence intensity was revealed by flow cytometry. In contrast, an insignificant shift in fluorescence intensity was observed at 4°C, being less than 5% of the binding observed at 37°C. Binding of FITC-HDL_3 to monocytes was temperature-dependent, consistent with the findings of Kagami, Fidge, and Nestel (25) and Morrison, McPherson, and Fidge (26), who showed that temperature had a marked effect on the association of ^{125}I-labeled HDL_3 with cells or rat liver plasma membranes. Fluorescent grains were visible on the surface and in the cytoplasm of monocytes after incubation with FITC-HDL_3 at 37°C for 1 h, but not after incubation at 4°C (data not shown), confirming this effect.

Specificity of the interaction was studied. Recently, it was reported that the human CLA-1 expressed in human monocytes was a high-affinity specific receptor for HDL, native LDL, and modified LDL (3). The results from the competition experiment indicated that LDL inhibited binding of ^{125}I-labeled HDL by the CLA-1-transfected cells as efficiently as unlabeled HDL. In contrast, our results showed that monocytes did not bind FITC-CD4 (a lymphocyte surface marker) or free FITC, and the FITC-HDL_3 binding was displaced by excess HDL_3 but not by VLDL or LDL. FITC-HDL_3 binding was reduced less in the presence of HDL_2 than HDL_3, and the competition curves for HDL_2 and HDL_3 differed. Thus the binding site was specific for HDL_3 with some cross-reactivity with HDL_2, and is possibly related to the apolipoprotein constituents, apoA-I and apoA-II, that are common to both HDL_3 and HDL_2, but present in different proportions. Both proteins have been shown to be capable of facilitating HDL binding to cells, membranes, and isolated HDL binding proteins (1, 4). However, it is not yet certain to what extent the lipid or apolipoprotein composition (i.e., the apoA-I/apoA-II or protein/lipid ratios) of HDL_3 or HDL_2 influences their ability to compete for cell surface binding. Importantly, FITC-labeled apo-HDL_3 bound to monocytes under the same conditions as HDL_3 and the results of ligand blotting support the presence of apo-HDL-specific binding sites in human blood monocytes. FITC-HDL_3 binding was almost eliminated by trypsin treatment, strengthening the involvement of cell surface receptors in the process. Further, some of the FITC-HDL_3 was apparently internalized by the cells after incubation at 37°C. These results suggest that FITC-HDL_3 is bound and internalized by a specific binding site.

Monocyte-derived macrophages are involved in the formation of early atherosclerotic lesions (27). Macrophages possess the scavenger receptor (28–31) that recognizes certain modified forms of LDL, including acetylated or oxidized LDL. Previous reports also suggest that HDL may bind specifically to coated pits in surface membranes of rat peritoneal macrophages (32). It has been suggested that candidate HDL receptors may be involved in the efflux of cholesterol from macrophage-derived cells. Thus,

monocytes are, potentially, very useful for probing the involvement of cell receptors in HDL metabolism.

The HDL-binding proteins from human mononuclear cells were partially purified by DEAE-Sephacel chromatography. Ligand blotting showed the presence of two major HDL-binding proteins of approximately 100 kDa and 120 kDa and a minor band of approximately 110 kDa. Thus, these proteins appear to correspond to the two HDL-binding proteins, 120 kDa HB_1 and 100 kDa HB_2, purified previously from rat liver plasma membranes, suggesting that HDL processing by both monocytes and liver cells occurs via common pathways. There was little evidence, from ligand blots, of an HDL-binding protein that corresponded to SR-B1 (M_r 80,000) and CLA-1 (M_r 83,000). The sequence of an HDL-binding protein from cultured bovine aortic endothelial cells was previously reported by McKnight et al. (33), but the characteristics of this cellular HDL-binding protein (HBP) differ from HB_1 and HB_2 purified in our laboratory, one of which (HB_2) shows significant homology with members of the immunoglobulin superfamily (16). However, further purification followed by the sequencing of both the 100 kDa and 120 kDa monocyte proteins is required to confirm the identity and structure of these two binding proteins.

The results of the present study confirm that monocytes possess specific HDL_3-binding sites, and at least two HDL-binding proteins. A combination of flow cytometry, FITC-labeled HDL_3, and a blood sample provides a simple assay for the expression of the HDL receptors HB_1 and HB_2 in human subjects, and the potential to survey the interaction between HDL metabolism, composition, and receptors in various forms of lipoproteinemia.

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