Cell surface-expressed moesin-like HDL/apoA-I binding protein promotes cholesterol efflux from human macrophages

Akifumi Matsuyama,* Naohiko Sakai,† Hisatoyo Hiraoka,† Ken-ichi Hirano,† and Shizuya Yamashita†,[1]

Medical Center for Translational Research,* Osaka University Hospital, 2-15 Yamada-oka, Suita; and Department of Cardiology,† Osaka University Graduate School of Medicine, B5, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan

Abstract **HDL and its major component, apolipoprotein A-I (apoA-I), play a central role in reverse cholesterol transport. We recently reported the involvement of a glycosylphosphatidylinositol anchor (GPI anchor) in the binding of HDL and apoA-I on human macrophages, and purified an 80 kDa HDL/apoA-I binding protein. In the present study, we characterized the GPI-anchored HDL/apoA-I binding protein from macrophages. The HDL/apoA-I binding protein was purified from macrophages and digested with endopeptidase, and the resultant fragments were sequenced. Cholesterol efflux, flow cytometry, immunoblotting, and immunohistochemical analyses were performed to characterize the HDL/apoA-I binding protein. Two parts of seven amino acid sequences completely matched those of moesin. Flow cytometry, immunoblotting, and immunohistochemistry using anti-moesin antibody showed that the HDL/apoA-I binding protein was N-glycosylated and expressed on the cell surface. It was termed *moesin-like protein.* Treatment of macrophages with anti-moesin antibody blocked the binding of HDL/apoA-I and suppressed cholesterol efflux. The moesin-like protein was exclusively expressed on macrophages and was upregulated by cholesterol loading and cell differentiation. Our results indicate that the moesin-like HDL/apoA-I binding protein is specifically expressed on the surface of human macrophages and promotes cholesterol efflux from macrophages.—** Matsuyama, A, N. Sakai, H. Hiraoka, K-i. Hirano, and S. Yamashita. **Cell surface-expressed moesin-like HDL/apoA-I binding protein promotes cholesterol efflux from human macrophages. *J. Lipid Res.* 2006.** 47: **78–86.**

Supplementary key words apolipoprotein A-I • glycosylphosphatidylinositol-anchored protein • moesin-like protein • atherosclerosis

The risk of atherosclerosis is inversely correlated with plasma concentration of HDL-cholesterol (1). The most important mechanism through which HDL exerts its protective role against atherosclerosis is the removal of excess cholesterol from peripheral cells, especially from lipid-laden macrophages, and the transport of this excess cholesterol to the liver, a process called reverse cholesterol transport (2). There are at least two mechanisms in the initial step of this system (3). The first is a passive aqueous diffusion mechanism, by which cholesterol desorbes from the plasma membrane pool to extracellular phospholipid-containing acceptor particles (e.g., HDL) via a concentration gradient between the membrane and acceptors. The second mechanism involves the interaction between HDL/apolipoprotein A-I (apoA-I) and specific binding sites on the cell surface, which induces an intracellular signal leading to the translocation of cholesterol from intracellular sites to the plasma membrane and subsequent transport of cholesterol to extracellular lipid-poor acceptors (4).

A specific apo HDL/apoA-I binding to various cells has been shown by a number of investigators. Several candidate HDL/apoA-I binding proteins have been identified, such as scavenger receptor class B type I and ATP binding cassette transporter A1 (5–9). Cdc42Hs, a member of the Rho GTPase family, may also be implicated in the transport and efflux of cholesterol (10).

Recently, we succeeded in purifying a phosphatidylinositol-specific phospholipase C (PI-PLC)-sensitive 80 kDa protein that is involved in HDL/apoA-I binding and cholesterol efflux from human monocyte-derived macrophages (11). In this follow-up study, we characterized the HDL/apoA-I binding protein and demonstrated that it is a moesin-like protein expressed on the cell surface.

Manuscript received 21 September 2004 and in revised form 26 September 2005 and in re-revised form 25 October 2005.

Published, JLR Papers in Press, October 26, 2005.
DOI 10.1194/jlr.M500425-JLR200

Abbreviations: AcLDL, acetylated LDL; apoA-I, apolipoprotein A-I; GPI, glycosylphosphatidylinositol; IEF, isoelectric focusing; MIF, mean intensity of fluorescence; PI-PLC, phosphatidylinositol-specific phospholipase C.

[1] To whom correspondence should be addressed.
e-mail: shizu@imed2.med.osaka-u.ac.jp

Anti-moesin antibody decreased the binding and subsequent cholesterol efflux of HDL/apoA-I from macrophages, suggesting that the moesin-like protein is involved in the efflux of cholesterol from human macrophages.

MATERIALS AND METHODS

Amino acid sequencing

The purified 80 kDa apoA-I/HDL binding protein (11) was applied for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and extracted directly from the gel by an in-gel lysil-endopeptidase digestion method. The digested fragments were fractionated by high-performance liquid chromatography, and seven peaks were individually collected. They were subjected to amino acid sequencing in a Perkin-Elmer sequencer, and two amino acid sequences were identified.

Isolation of human monocyte-derived macrophages

Mononuclear cells were isolated from the buffy coats of blood collected from healthy volunteers using density gradient centrifugation with Lymphoprep (Nycomed; Oslo, Norway) (12) and cultured as described previously (11). After a 6 day culture, macrophages were loaded with cholesterol by incubation with 50 μg/ml of acetylated LDL (AcLDL), and then used for the experiments.

Cell culture

HepG2 and HEK293 cells, human fibroblasts, and THP-1 cells were obtained from American Type Cell Collection. HepG2 and HEK293 cells and human fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS). THP-1 cells were cultured in RPMI 1640 supplemented with 10% FCS and were transformed to macrophages by incubation for 24 h with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Nacalai Tesque; Kyoto, Japan).

Lipoprotein isolation, modification, and labeling

HDL3 was isolated from human plasma by ultracentrifugation at a density of 1.125–1.210 g/ml (13). Protein concentration was measured following the procedure of Lowry et al. (14). ApoA-I was purchased from Sigma (St. Louis, MO). HDL3 and apoA-I were labeled with fluorescein isothiocyanate (FITC) (Molecular Probes; Eugene, OR) as reported previously (11, 15). DiI-AcLDL was purchased from Molecular Probes. DiI-oxidized LDL was from Biomedical Technologies, Inc. (Stoughton, MA).

Immunoblotting analysis

Plasma membranes were prepared from human monocyte-derived macrophages, HepG2 cells, HEK293 cells, and human fibroblasts as described previously (11), and were applied for immunoblotting analysis with anti-moesin antibody (TK88, kind gift from S. Tsukita, Kyoto University Graduate School of Medicine). For two-dimensional immunoblotting analysis, the membrane proteins were separated using isoelectric focusing (IEF) gels (Amersham Pharmacia Biotech; Buckinghamshire, UK) according to the protocol supplied by the manufacturer and 4–20% polyacrylamide gradient gels (Daiichi Pure Chemicals; Tokyo, Japan), transferred onto nitrocellulose membranes, and blotted with anti-moesin antibody (TK88). After incubation with peroxidase-conjugated anti-rabbit IgG antibody, the blots were visualized with an ECL kit (Amersham Pharmacia Biotech).

PI-PLC treatment

To determine the effect of PI-PLC (Sigma), the macrophages were incubated at 37°C with 1 U/ml of PI-PLC for 1 h and applied for experiments.

Enzymatic deglycosylation

Concentrated PI-PLC-treated media (10 μl) were incubated for 24 h at 37°C with or without 50 units of N-glycosidase F (Sigma) in potassium buffer (200 mM K_3PO_4, 20 mM EDTA, pH 7.2) as indicated by the manufacturer, followed by the addition of 1 vol of 2× Laemmli sample buffer, and applied for immunoblotting analysis.

Flow cytometry

Cell surface expression of moesin-like protein was confirmed by flow cytometric analysis using anti-moesin antibody (TK88 and TK89, a kind gift from S. Tsukita). Competition analyses with anti-moesin antibody or control IgG were performed by incubating cells for 1 h at 4°C with FITC-labeled HDL3 or apoA-I. All fluorescence measurements were performed by flow cytometry using a FACScan (Becton-Dickinson; Mountain View, CA). Cell-bound fluorescence intensity was expressed as the mean intensity of fluorescence (MIF).

Immunohistochemistry

For immunohistochemical analysis to detect moesin, cultured human macrophages were incubated in a 2-well glass chamber slide (Falcon) and fixed for 30 min with 4% paraformaldehyde. The cells were blocked with 5% normal horse serum and incubated with anti-moesin antibodies (TK88 and TK89) in PBS containing 3% BSA for 1 h under detergent-free conditions, followed by sequential incubation with FITC-labeled anti-rabbit IgG antibody. The treated cells were observed using confocal fluorescence microscopy (Floview FV1000; Olympus, Tokyo).

Cell surface biotinylation

The cholesterol-laden macrophages and fibroblasts were washed with ice-cold phosphate buffer (10 mM, pH 7.4). They were subsequently treated with 1 ml of 0.8 mM NHS-biotin (water-soluble biotin; Pierce, Rockford, IL) in phosphate buffer for 15 min on ice, and washed three times with 0.192 M glycine-25 mM Tris (pH 8.3) to quench the reaction. The cells were lysed in MES buffer containing a cocktail of protease inhibitors (0.5 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A; Roche Applied Science), and membrane fractions were obtained from the materials as described previously (11). The membrane pellets were dissolved and applied for immunoprecipitation using anti-moesin antibody and control IgG. Immunoprecipitates were applied for SDS-PAGE and visualized with streptavidin-horseradish peroxidase (HRP) and the ECL kit.

Measurement of cellular cholesterol efflux from macrophages

Cellular cholesterol efflux via HDL3 or apoA-I was determined as described previously (11, 16). The cells were incubated with 20 μg/ml of HDL3 or 5 μg/ml of apoA-I diluted in RPMI with 5 mg/ml of BSA with 100 μg/ml of anti-moesin antibody or control IgG (Transduction Laboratories; San Jose, CA). After incubation for the indicated time, radioactivities in both the medium and the cells were measured separately. Fractional cholesterol efflux was calculated as the amount of radioactivity in the medium divided by the total radioactivity in each well and expressed as a percentage. The cellular cholesterol contents were

measured using a cholesterol-measuring kit (Kyowa Medics, Tokyo) according to the instructions provided by the manufacturer.

RESULTS

Sequencing of the 80 kDa HDL/apoA-I binding protein of macrophages

A single band of the 80 kDa apoA-I/HDL binding protein (5 pmol) was obtained from human macrophages derived from 25 healthy volunteers (10 l of whole blood) as reported previously (11), and applied for lysil-endopeptidase digestion followed by fractionation using HPLC. Seven peaks were individually collected and subjected to amino acid sequencing in a Perkin-Elmer sequencer. Two of them were successfully sequenced. The sequences of the peptides (DQWEERIQVWH and TANDEMHAEN) were the same as the internal sequences of human moesin (amino acid 164–174 and 540–549, respectively) (17).

Designation of the 80 kDa HDL/apoA-I binding protein as moesin-like protein

Previous studies reported that moesin is localized in the sub-membranous cytoskeleton, filopodia, and other membranous protrusions (17), but to our knowledge, cell surface expression of moesin has not been reported previously. To determine whether the HDL/apoA-I binding protein is identical to moesin, we first performed immunoblotting analyses using anti-moesin antibody (**Fig. 1**). The 80 kDa moesin-like protein was recovered in the conditioned media after PI-PLC treatment, whereas no immunoreactive mass was observed without PI-PLC treatment (Fig. 1Aa). It was noted that the molecular weight of the moesin-like protein was about 2 kDa larger than that located in the cell membrane. To confirm the loss of the moesin-like protein from the membrane after treatment with PI-PLC, membrane fractions of macrophages with or without PI-PLC treatment were applied for immunoblotting with anti-moesin antibody (Fig. 1Ab). The larger bands of two moesin-like proteins were reduced after PI-PLC treatment. These results suggest that the protein is a glycosylphosphatidylinositol (GPI)-anchored type and not identical to moesin.

To confirm that the 80 kDa HDL/apoA-I binding protein is not identical to moesin, two-dimensional immunoblotting was performed (Fig. 1B). The membrane fraction of human macrophages and the conditioned media after PI-PLC treatment were applied for two-dimensional immunoblotting with anti-moesin antibody. A massive amount of moesin at pH 6.0 (asterisk), as predicted by the amino acid content of moesin, and a small amount of protein with isoelectric points of pH 7.2 to 7.6 (arrowheads) were observed in membrane fraction proteins. The conditioned media with PI-PLC treatment showed moesin-like protein (arrowheads) but not moesin.

To further confirm that the 80 kDa HDL/apoA-I binding protein was not identical to moesin, enzymatic deglycosylation with N-glycosidase F was performed. After deglycosylation with the enzyme, the molecular weight of the 80 kDa HDL/apoA-I binding protein was shifted down compared with that of the nondigested protein (Fig. 1Ca), indicating that the protein was N-glycosylated. Next, the change in isoelectric point of the protein after deglycosylation was examined. If the protein was N-linked, charged glucans would be detached after deglycosylation and the isoelectric point would change. After N-glycosidase F treatment (Fig. 1Cb), the isoelectric point of the protein (pH 7.2 to 7.6) changed to pH 6.0. This result reinforced the notion that the protein was N-glycosylated. Because moesin is not expressed on the cell surface, as reported previously (17), the 80 kDa HDL/apoA-I binding protein was confirmed to be different from moesin and thus designated as moesin-like protein.

Expression of moesin-like protein on the cell surface of human macrophages

To determine whether the moesin-like protein was expressed on the cell surface of human macrophages, flow cytometry was performed using anti-moesin antibodies under detergent-free conditions. As shown in Fig. 2Aa, moesin-like protein was detected on the surface of macrophages by anti-moesin antibodies (TK88 and TK89). To confirm the loss of moesin-like protein from the cell surface, flow cytometric analysis was performed after PI-PLC treatment (Fig. 2Ab). PI-PLC treatment shifted the distribution to the left, indicating that the moesin-like protein was GPI-anchored and expressed on the cell surface.

To observe the distribution of the cell surface-expressed moesin-like protein, confocal microscopic analysis was performed using anti-moesin antibody (TK88) under detergent-free conditions (**Fig. 2B**). *En face* sections (*xy*) images were taken off the macrophage from the top to the bottom, and computer-reconstructed vertical section (*xz* and *yz*) images taken through the full thickness of the macrophage were obtained. Moesin-like immunoreactivities were observed only on the cell surface of the macrophages. No fluorescence signal was observed using control rabbit IgG (data not shown).

To further confirm that human monocyte-derived macrophages express moesin-like protein on their cell surface, cell surface biotinylation analysis was performed (Fig. 2C). After a monolayer of macrophages was treated with NHS-biotin (water-soluble biotin), the membrane fraction of the cells was collected and immunoprecipitated with anti-moesin antibody (TK88). The immunoprecipitates were applied for SDS-PAGE, transferred onto nitrocellulose membrane, and then visualized with avidin-HRP. As indicated by the arrow (Fig. 2C), the 80 kDa protein was labeled with NHS-biotin, suggesting that the moesin-like protein was expressed on the cell surface of macrophages. However, the biotinylated protein was not visualized on the fibroblasts, indicating that the expression of moesin-like protein is specific for macrophages.

Effect of anti-moesin antibody on HDL/apoA-I binding and HDL/apoA-I-mediated cholesterol efflux from macrophages

To determine whether moesin-like protein is an HDL/apoA-I binding protein, competition studies with anti-moesin

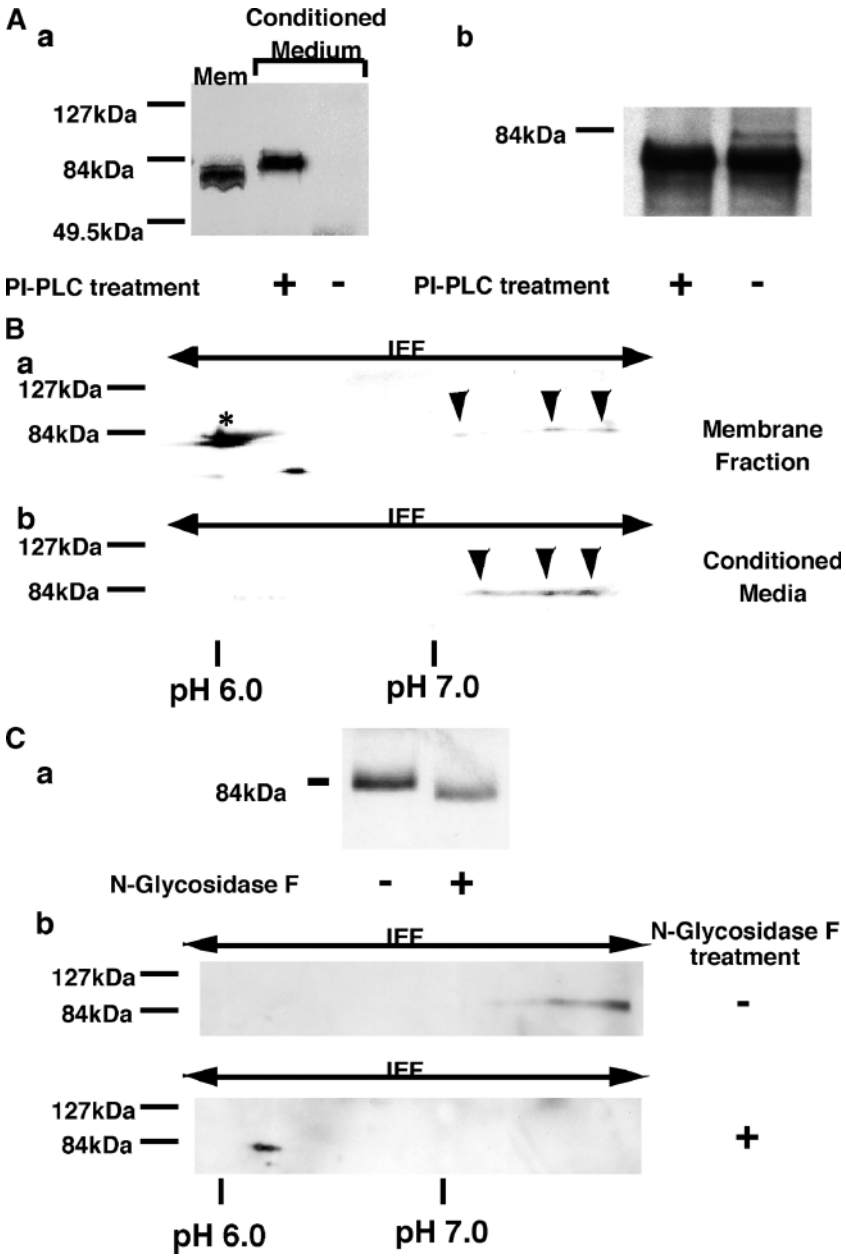

Fig. 1. Moesin-like protein is an 80 kDa HDL/apolipoprotein A-I (apoA-I) binding protein. A: Formation of moesin-like protein by phosphatidylinositol-specific phospholipase C (PI-PLC) treatment of human macrophages. After treatment of macrophages with or without 0.5 U/ml PI-PLC, the conditioned media were applied for immunoblotting analysis with anti-moesin antibody (TK88) (a). PI-PLC treatment resulted in the formation of the moesin-like protein from human macrophages. In control experiments (incubation without PI-PLC), no band was observed. After treatment with or without PI-PLC, the macrophage membranes were subjected to immunoblotting with anti-moesin antibody (b). The larger band of two moesin-like proteins was reduced after PI-PLC treatment. B: Two-dimensional immunoblotting using anti-moesin antibody. The membrane fraction of human macrophages (a) and the conditioned media after PI-PLC treatment with macrophages (b) were applied for two-dimensional immunoblotting analysis with anti-moesin antibody (TK88). A massive amount of moesin at pH 6.0 (asterisk), as predicted by the amino acid content of moesin, and a small amount of protein with an isoelectric point of pH 7.2 to 7.6 (arrowheads) were observed in membrane fraction proteins. The conditioned media with PI-PLC treatment showed moesin-like protein (arrowheads) but not moesin. C: Enzymatic deglycosylation of moesin-like protein with N-glycosidase F. The conditioned media, after treatment with 0.5 U/ml PI-PLC for 24 h at 37°C, were incubated with or without N-glycosidase F. After digestion with the enzyme, the treated or nontreated conditioned media were applied for immunoblotting with anti-moesin antibody (TK88). The immunoreactive protein of digested media was shifted down compared with that of nondigested media (a), and the isoelectric point of the protein (pH 7.2 to 7.6) changed to pH 6.0 (b), indicating that the protein was N-linked.

antibody were performed using flow cytometry (**Fig. 3A**). Compared with control IgG, anti-moesin monoclonal antibody reduced the MIF of FITC-labeled HDL3 from 46.0 to 21.9 (Fig. 3Aa) and that of FITC-labeled apoA-I from 203.2 to 80.0 (Fig. 3Ab). To confirm that anti-moesin antibody could specifically reduce HDL3 or apoA-I binding, we examined whether the antibody could suppress the bindings of DiI-oxidized LDL and DiI-AcLDL (Fig. 3Ac,

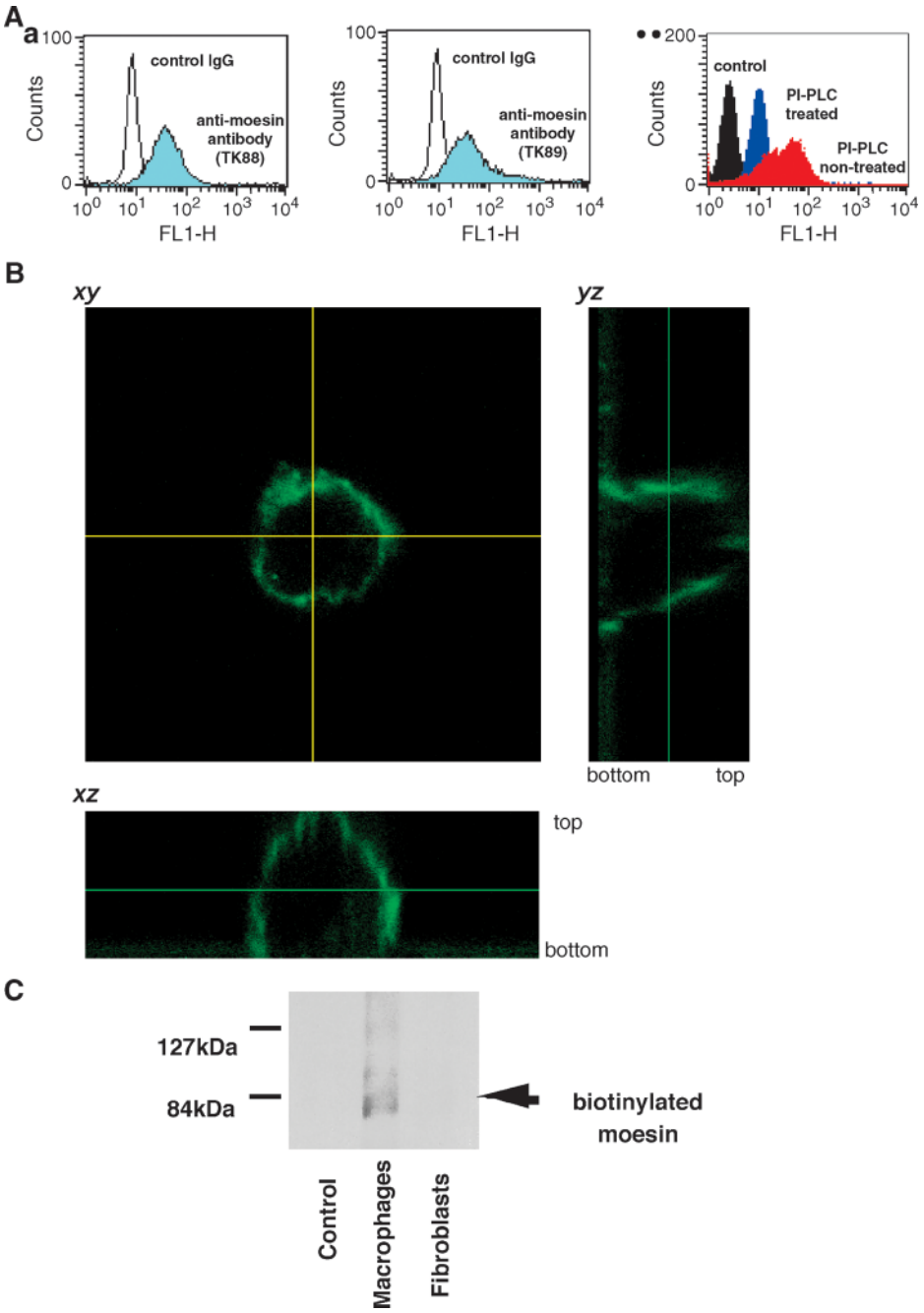

Fig. 2. Expression of moesin-like protein is on the cell surface of macrophages. A: Flow cytometric analyses of moesin-like protein on macrophages. After fixation with 4% paraformaldehyde, the lipid-laden macrophages were preincubated with 5% normal horse serum and then with control rabbit IgG or anti-moesin antibody (TK88 or TK89) (a). After washing, the cells were incubated with FITC-labeled goat anti-rabbit IgG antibody. Cell-bound fluorescence was detected by FACScan. After treatment with or without PI-PLC, the macrophages were applied for FACScan analyses with anti-moesin antibody (TK88) (b). PI-PLC treatment reduced the fluorescence of the moesin-like moiety. Red peak, PI-PLC nontreated macrophages; blue peak, PI-PLC-treated; black peak, control. B: Confocal microscopic analysis of moesin-like protein. For immunohistochemical analysis of moesin-like protein, cultured human macrophages were fixed with 4% paraformaldehyde, blocked with 5% normal horse serum, and then incubated with anti-moesin antibody (TK88) for 1 h, followed by sequential incubation with FITC-labeled anti-rabbit IgG antibody, and finally confocal microscopic analysis. Vertical sections (*xz* and *yz*) were projected. *En face* sections (*xy*) images were taken off the macrophage from the top to the bottom and computer-reconstructed vertical section (*xz* and *yz*) images were taken through the full thickness of the macrophage. C: Immunoprecipitation after cell surface biotinylation. The cultured macrophages and human fibroblasts were treated with NHS-biotin (water-soluble biotin) for 15 min at 4°C and then washed with 0.192 M glycine/25 mM Tris, pH 8.3, for 15 min at 4°C to quench the reaction. The membrane fractions were subjected to immunoprecipitation using anti-moesin antibody (TK88) and control IgG. Immunoprecipitates were applied for SDS-PAGE, transferred onto nitrocellulose membrane, and then visualized with streptavidin-horseradish peroxidase.

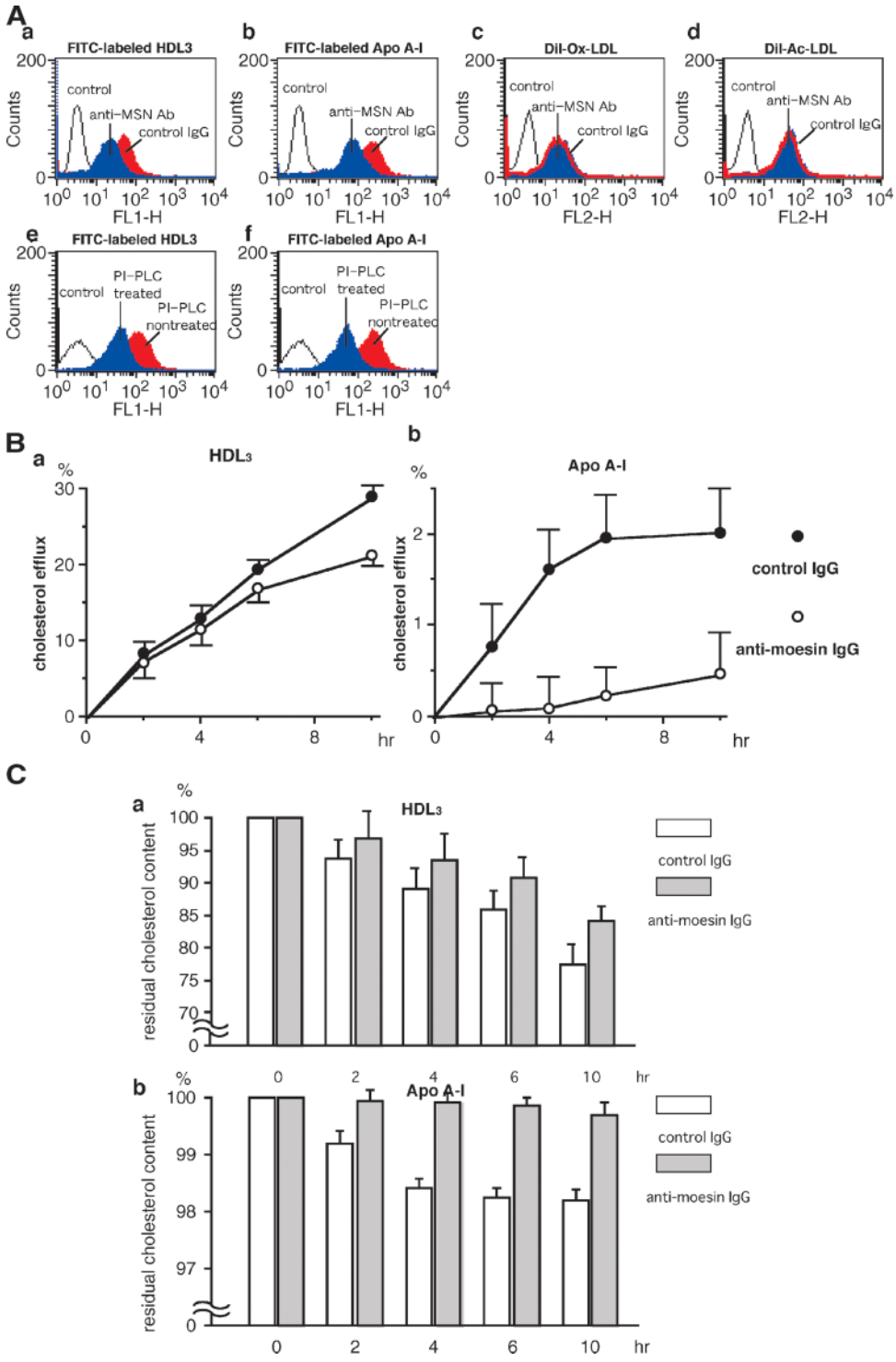

Fig. 3. Effects of anti-moesin antibody on HDL/apoA-I binding and HDL/apoA-I-mediated cholesterol efflux from macrophages. A: Flow cytometric analysis of HDL3/apoA-I-specific binding to macrophages via moesin-like protein. The effect of anti-moesin antibody on FITC-labeled HDL3 (a) and apoA-I (b) examined by flow cytometry. Anti-moesin antibody reduced the mean intensity of fluorescence (MIF), decreased HDL3 binding from 46.02 to 21.87, and apoA-I binding from 203.24 to 80.02. However, anti-moesin antibody did not affect DiI-labeled oxidized LDL (c) or DiI-labeled AcLDL (d). After PI-PLC treatment, binding studies were performed to determine the percentage of apoA-I (e) or HDL3 (f) binding due to this specific receptor. PI-PLC reduced the binding capacity of HDL3 by 67% and that of apoA-I by 76%. B: Anti-moesin antibody inhibits HDL3/apoA-I-mediated cholesterol efflux. Cholesterol efflux was evaluated as the transfer of [^{3}H]-labeled cholesterol from prelabeled macrophages to HDL3 (a) or apoA-I (b). The percentage of specific HDL3- or apoA-I-mediated cholesterol efflux was estimated by the difference between cholesterol efflux in the presence and absence of the ligands. Anti-moesin antibody decreased cholesterol efflux via HDL3 (a) and apoA-I (b) from macrophages throughout the experiment compared with the control IgG. C: Effect of anti-moesin antibody on residual cholesterol content in macrophages after HDL3 (a) or apoA-I (b) -mediated cholesterol efflux. Anti-moesin antibody suppressed the reduction of residual cholesterol after cholesterol efflux throughout the experiment. Data are mean ± SEM of n experiments.

Ad). Neither the distribution of DiI-oxidized LDL nor that of DiI-AcLDL-incorporated cells was affected by incubation with anti-moesin antibody. Next, to address the percentage of apoA-I or HDL binding due to this specific receptor, binding studies were performed using PI-PLC-treated cells (Fig. 3Ae and f). After treatment with PI-PLC, the binding capacity of HDL3 diminished from 121.5 to 39.5 (67% reduction) and that of apoA-I from 229.1 to 55.4 (76% reduction).

To confirm the physiological function of moesin-like protein on the cell surface, we next examined whether it was involved in the HDL/apoA-I-mediated cholesterol efflux from macrophages. As shown in Fig. 3B, treatment of macrophages with anti-moesin antibody reduced HDL- and apoA-I-mediated cholesterol efflux from macrophages throughout the experiment (up to 10 h). Next, we examined the effect of anti-moesin antibody on the residual cholesterol contents of macrophages after HDL- or apoA-I-mediated cholesterol efflux (Fig. 3C). Anti-moesin antibody lessened the reduction of residual cholesterol after cholesterol efflux. These results suggest that the moesin-like protein is involved in HDL- and apoA-I-mediated cholesterol efflux from human monocyte-derived macrophages.

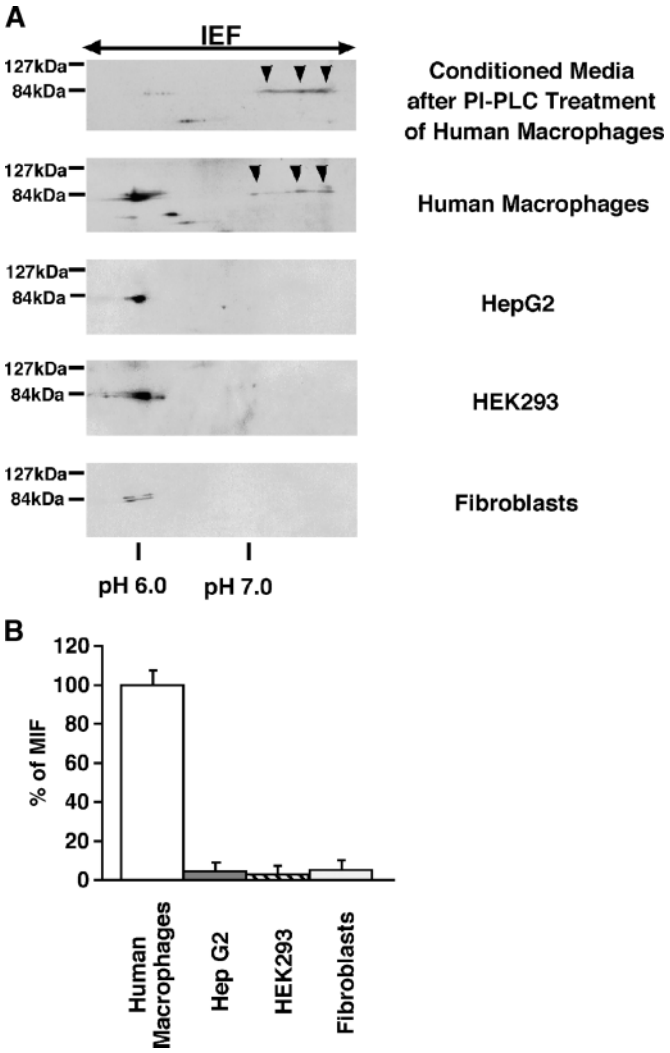

Fig. 4. Specific expression of the moesin-like protein on human macrophages. A: Cell lineage-specific expression of the moesin-like protein in two-dimensional immunoblotting. Membrane fractions derived from human macrophages, HepG2 cells, HEK293 cells, and human fibroblasts were subjected to two-dimensional immunoblotting analysis using anti-moesin antibody (TK88). Human macrophages expressed the moesin-like protein (arrowheads) in addition to moesin. Nonmonocyte-lineage cells (HepG2, HEK293, and human fibroblasts) expressed moesin but not moesin-like protein. B: Cell lineage-specific expression of the moesin-like protein by flow cytometry. Human macrophages, THP-1, HepG2 and HEK293 cells, and human fibroblasts were subjected to flow cytometry. The moesin-like protein was not detected in nonmonocyte-lineage cells (HepG2, HEK293, and human fibroblasts), but was expressed in human macrophages and THP-1 cells, suggesting that moesin-like protein is specifically expressed on monocyte-macrophages lineage. Data are mean ± SEM of n experiments. IEF, isoelectric focusing.

Specific expression of moesin-like protein in monocytes/macrophages

To determine whether the cell surface-expressed moesin-like protein is specifically expressed on macrophages, two-dimensional immunoblotting was performed (**Fig. 4A**). Membrane fractions from human monocyte-derived macrophages, HepG2 cells, HEK293 cells, and human fibroblasts were collected and applied for two-dimensional immunoblotting with anti-moesin antibody (TK88). Human macrophages expressed moesin with an isoelectric point of pH 6.0 and moesin-like protein with an isoelectric point of pH 7.2 to 7.6. Cells other than monocyte macrophages expressed moesin (pH 6.0) but no moesin-like protein.

The moesin-like protein on the cell surface was further examined by flow cytometry, and its amount was determined as MIF in human macrophages, HepG2 cells, HEK293 cells, and human fibroblasts (Fig. 4B). The results confirmed the lack of moesin-like protein on the surface of nonmonocyte cell lines (HepG2, HEK293, and human fibroblasts) and its presence on human macrophages.

Effect of cholesterol loading and differentiation on the expression of moesin-like protein

To determine the functional role of the cell surface-expressed moesin-like protein in the reverse cholesterol transport system, we examined the effect of cholesterol loading. Six-day-cultured human macrophages were incubated for 24 h with or without AcLDL (50 μg/ml), and then subjected to flow cytometric analysis. The distribution of cell surface moesin-positive macrophages was shifted to the right by Ac-LDL loading (**Fig. 5Aa**), and the MIF of AcLDL-laden macrophages was upregulated about two-fold to that of unladen macrophages (Fig. 5Ab). To confirm that the moesin-like protein was increased after cholesterol loading, membrane fractions derived from Ac-LDL-laden and -unladen macrophages were applied for immunoblotting analyses using anti-moesin antibody (Fig. 5B). The larger band of two moesin-like proteins, which was shown as the cell surface-expressed moesin-like protein, was increased by cholesterol loading. These results suggest that the expression of cell surface-expressed moesin-like protein is enhanced in association with foam cell formation.

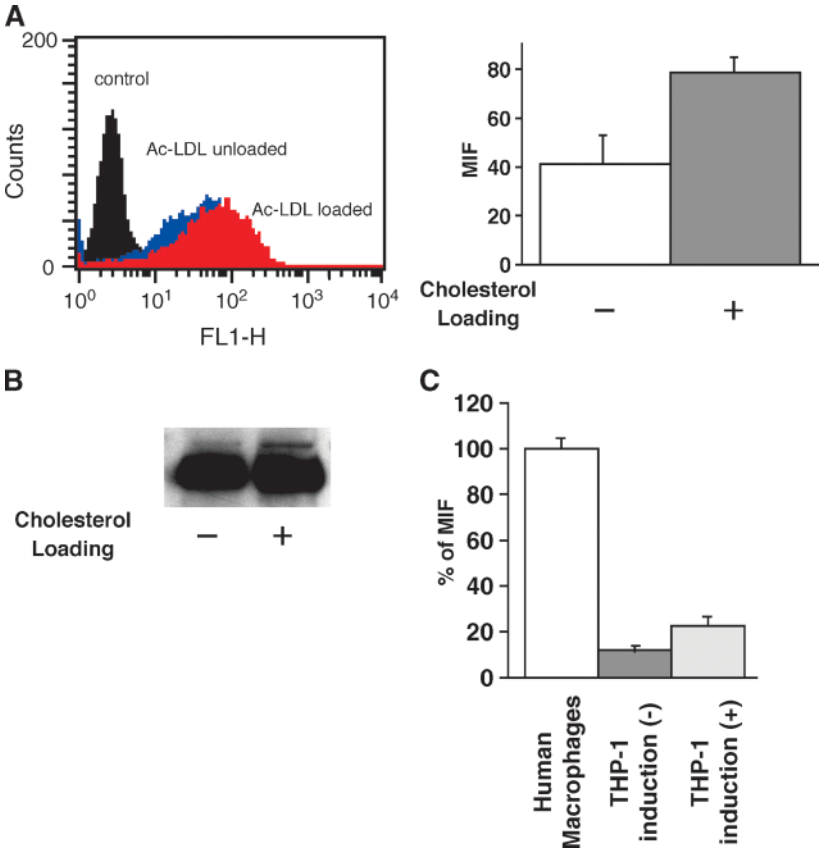

Fig. 5. A: Effect of cholesterol loading on moesin-like protein. Six day-cultured human macrophages were incubated for 24 h with or without AcLDL (50 μg/ml), and then subjected to flow cytometry. The MIF of AcLDL-laden macrophages was upregulated about two-fold relative to that of unladen macrophages. B: After loading cholesterol with Ac-LDL, the macrophage membranes were subjected to immunoblotting with anti-moesin antibody. The intensity of the larger band of two moesin-like proteins was increased on Ac-LDL-laden macrophages compared with nonladen. C: Effect of differentiation on moesin-like protein expression. THP-1 cells were treated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) for 24 h and then subjected to flow cytometry. The MIF of the moesin-like protein after PMA treatment was double that before treatment. Data are mean ± SEM of n experiments.

Finally, we examined whether moesin-like protein increases with cell differentiation. For this purpose, THP-1 cells were treated with PMA and subjected to flow cytometric analysis (Fig. 5C). The MIF of moesin-like protein after PMA treatment was expressed twice as much as that before treatment, indicating that the cell surface-expressed moesin-like protein was upregulated with differentiation of monocytes into macrophages.

DISCUSSION

In the present study, we identified the protein recognized by anti-human moesin antibodies, and termed it moesin-like protein. The protein is a GPI-anchored type, is expressed on the cell surface of human macrophages, is induced by differentiation and cholesterol loading of macrophages, and is involved in the binding and cholesterol efflux of HDL/apoA-I.

Internal sequencing of the previously purified apoA-I/HDL binding protein matched the sequences of the two internal fragments of moesin. Moesin belongs to the ERM family of proteins (along with ezrin and radixin) that are known to be involved in cell adhesion and membrane dynamics, probably because of their ability to link plasma membrane components with the actin cytoskeleton (17–19). Although the functions of ERM proteins have not yet been fully delineated, these proteins are widely expressed in different tissues and cells. They have been identified in filopodia and are associated with other membranous proteins that are important for ligand recognition, signal transduction, and cell motility (17–20). Most previous reports have focused on moesin as a linking protein of the submembranous cytoskeleton.

Although moesin has a cell surface domain involved in the binding of lipopolysaccharides (21), IL-2 fragments (22), and human immunodeficiency virus type 1 envelope protein gp 120 (23), moesin has no signal peptide, is not expressed on the cell surface, and is not a GPI-anchored type protein. In this study, we demonstrated that the newly purified apoA-I/HDL binding protein is expressed on the cell surface, exclusively on macrophages, and is a GPI-anchored type. Two-dimensional immunoblotting analysis also revealed that the IEF of the protein (pH 7.2–7.6) is different from that of moesin (pH 6.0). We also showed that the protein underwent N-glycosylation. These characteristics are totally different from those of moesin, indicating that the protein is not moesin.

There are no reports on isoforms of moesin. The protein is recognized by two kinds of antibodies raised by human moesin. The epitopes of these antibodies are located between two internal sequences that matched the fragments sequences of the moesin-like protein. The RT-PCR product, with primers located on two matched sequences using RNAs from human macrophages, yielded a single band (data not shown). These data suggest that the protein prified in our study is an isoform of moesin. The isoform of moesin is thought to be transported by the Golgi-ER

pathway and expressed on the cell surface of human macrophages. Gelsolin, for example, also has two isoforms; one functions as an actin binding protein intracellularly, and the other has a signal peptide and is secreted by the liver. The pattern of expression is also different for the two gelsolin isoforms. This alternative splicing mechanism of gelsolin resembles that of moesin. One of two isoforms of moesin can be a cell surface binding site of HDL and apoA-I.

In conclusion, our data demonstrate that the cell surface-expressed moesin-like protein is a binding protein for apoA-I/HDL on the surface of human monocyte-derived macrophages and can stimulate apoA-I- /HDL-mediated cholesterol efflux. Further studies are in progress to clone the cell surface-expressed moesin-like protein, which may enhance our understanding of the initial step of reverse cholesterol transport.

This research was supported in part by grants-in-aid (10671067, 11557054, 11557055, and 12670666) from the Japanese Ministry of Education, Culture, Sports and Welfare; a grant from the Kanae Foundation; a grant from the Japan Heart Foundation/Pfizer for research on hypertension, hyperlipidemia, and vascular metabolism (A.M.); and an International HDL Research Awards Program grant (S.Y.) from Pfizer.

REFERENCES

1. Gordon, T., W. P. Castelli, M. C. Hjortland, W. B. Kannel, and T. R. Dawber. 1977. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. *Am. J. Med.* **62:** 707–714.
2. Yamashita, S., N. Sakai, K. Hirano, T. Arai, M. Ishigami, T. Maruyama, and Y. Matsuzawa. 1997. Molecular genetics of plasma cholesteryl ester transfer protein. *Curr. Opin. Lipidol.* **8:** 101–110.
3. Oram, J. F., and S. Yokoyama. 1996. Apolipoprotein-mediated removal of cellular cholesterol and phospholipids. *J. Lipid Res.* **37:** 2473–2491.
4. Mendez, A. J., J. F. Oram, and E. L. Bierman. 1991. Protein kinase C as a mediator of high density lipoprotein receptor-dependent efflux of intracellular cholesterol. *J. Biol. Chem.* **266:** 10104–10111.
5. Matsumoto, A., A. Mitchell, H. Kurata, L. Pyle, K. Kondo, H. Itakura, and N. Fidge. 1997. Cloning and characterization of HB2, a candidate high density lipoprotein receptor. Sequence homology with members of the immunoglobulin superfamily of membrane proteins. *J. Biol. Chem.* **272:** 16778–16782.
6. Acton, S., A. Rigotti, K. T. Landschulz, S. Xu, H. H. Hobbs, and M. Krieger. 1996. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science.* **271:** 518–520.
7. Ji, Y., B. Jian, N. Wang, Yu Sun, M. L. Moya, M. C. Phillips, G. H. Rothblat, J. B. Swaney, and A. R. Tall. 1997. Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J. Biol. Chem.* **272:** 20982–20985.
8. Hirano, K., S. Yamashita, Y. Nakagawa, T. Ohya, F. Matsuura, K. Tsukamoto, Y. Okamoto, A. Matsuyama, K. Matsumoto, J. Miyagawa, et al. 1999. Expression of human scavenger receptor class B type I in cultured human monocyte-derived macrophages and atherosclerotic lesions. *Circ. Res.* **85:** 108–116.
9. Wang, N., D. L. Silver, P. Costet, and A. R. Tall. 2000. Specific binding of Apo A-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. *J. Biol. Chem.* **275:** 33053–33058.
10. Hirano, K., F. Matsuura, K. Tsukamoto, Z. Z. Zhang, A. Matsuyama, K. Takaishi, R. Komuro, T. Suehiro, S. Yamashita, and Y. Takai. 2000. Decreased expression of a member of the Rho GTPase family, Cdc42Hs, in cells from Tangier disease - the small G protein may play a role in cholesterol efflux. *FEBS Lett.* **484:** 275–279.
11. Matsuyama, A., S. Yamashita, N. Sakai, T. Maruyama, E. Okuda, K. Hirano, S. Kihara, H. Hiraoka, and Y. Matsuzawa. 2000. Identification of a GPI-anchored type HDL-binding protein on human macrophages. *Biochem. Biophys. Res. Commun.* **272:** 864–871.
12. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand. J. Clin. Lab. Invest.* **97 (Suppl.):** 77–89.
13. Havel, R. J., H. Eder, and J. H. Bradgon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **3:** 1345–1353.
14. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
15. Takata, K., S. Horiuchi, A. T. Rahim, and Y. Morino. 1988. Receptor-mediated internalization of high density lipoprotein by rat sinusoidal liver cells: identification of a nonlysosomal endocytic pathway by fluorescence-labeled ligand. *J. Lipid Res.* **29:** 1117–1126.
16. de la Llera Moya, M., V. Atger, J. L. Paul, N. Fournier, N. Moatti, P. Giral, K. E. Friday, and G. Rothblat. 1994. A cell culture system for screening human serum for ability to promote cellular cholesterol efflux. Relations between serum components and efflux, esterification, and transfer. *Arterioscler. Thromb. Vasc. Biol.* **14:** 1056–1065.
17. Sato, N., N. Funayama, A. Nagafuchi, S. Yonemura, S. Tsukita, and S. Tsukita. 1992. A gene family consisting of ezrin, radixin, and moesin: its specific localization at actin filament/plasma membrane association sites. *J. Cell Sci.* **103:** 131–143.
18. Sagara, J., S. Tsukita, S. Yonemura, S. Tsukita, and A. Kawai. 1995. Cellular actin-binding ezrin-radixin-moesin (ERM) family proteins are incorporated into the rabies virion and closely associated with viral envelope proteins in the cell. *Virology.* **206:** 485–494.
19. Mangeat, P., C. Roy, and M. Martin. 1999. ERM proteins in cell adhesion and membrane dynamics. *Trends Cell Biol.* **9:** 187–192.
20. Nakamura, F., L. Huang, K. Pestonjamasp, E. J. Luna, and H. Furthmayr. 1999. Regulation of F-actin binding to platelet moesin in vitro by both phosphorylation of threonine 558 and phosphosphatidylinositides. *Mol. Biol. Cell.* **10:** 2669–2685.
21. Tohme, Z. N., S. Amar, and T. E. van Dyke. 1999. Moesin functions as a lipopolysaccharide receptor on human monocytes. *Infect. Immun.* **67:** 3215–3220.
22. Ariel, A., R. Hershkoviz, I. Altbaum-Weiss, S. Ganor, and O. Lider. 2001. Cell surface-expressed moesin-like receptor regulates T cell interactions with tissue components and binds an adhesion-modulating IL-2 peptide generated by elastase. *J. Immunol.* **166:** 3052–3060.
23. Hecker, C., C. Weise, J. Schneider-Schaulies, H. C. Holmes, and V. ter Meulen. 1997. Specific binding of HIV-1 envelope protein gp120 to the structural membrane proteins ezrin and moesin. *Virus Res.* **49:** 215–223.