Involvement of caveolin-1 in cholesterol enrichment of high density lipoprotein during its assembly by apolipoprotein and THP-1 cells

Reijiro Arakawa, Sumiko Abe-Dohmae, Michiyo Asai, Jin-ichi Ito, and Shinji Yokoyama[1]

Biochemistry 1, Nagoya City University Medical School, Kawasumi 1, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

Abstract **High density lipoprotein (HDL) is assembled by interaction of apolipoprotein A-I with human monocytic leukemia cell line THP-1 by removing cellular cholesterol and phospholipid. Although the HDL formed with undifferentiated THP-1 cells contained only phosphatidylcholine and almost no cholesterol, the cells differentiated with phorbol 12-myristate 13-acetate (PMA) generated HDL enriched in cholesterol. The extent of cholesterol enrichment related to the cellular cholesterol level in the differentiated cells, but only weakly in the undifferentiated cells. In contrast, the differentiation had no influence on the diffusion-mediated cellular cholesterol efflux. The undifferentiated cells expressed the messages of ATP-binding cassette transporter 1 and caveolin-1, at low levels, and the PMA-induced differentiation resulted in substantial expression of both messages. Caveolin-1 protein expression was also highly induced by the PMA treatment of THP-1 cells. When the cells were treated with the antisense DNA of caveolin-1 and differentiated, both caveolin-1 synthesis and cholesterol incorporation into the HDL were reduced in parallel to generate the cholesterol-poor HDL. We concluded that caveolin-1 is involved in enrichment with cholesterol of the HDL generated by the apolipoprotein-cell interaction. This function is independent of the assembly of HDL particles with cellular phospholipid and of nonspecific, diffusion-mediated efflux of cellular cholesterol.** —Arakawa, R., S. Abe-Dohmae, M. Asai, J-i. Ito, and S. Yokoyama. **Involvement of caveolin-1 in cholesterol enrichment of high density lipoprotein during its assembly by apolipoprotein and THP-1 cells.** ***J. Lipid Res.*** **2000.** 41: **1952–1962.**

Supplementary key words cholesterol efflux • ABC1 • PMA

Cholesterol is not metabolized in peripheral cells except for the limited catabolism by sterol 27-hydroxylase and steroidogenesis in certain specific organs. Most cellular cholesterol is therefore removed and transported to the liver for its conversion to bile acids. High density lipoprotein (HDL) is believed to play a central role in this pathway. Two major mechanisms are proposed for the initial step of this transport system (1); nonspecific diffusion of cholesterol out of the cells through the aqueous phase and assembly of HDL by apolipoprotein with cellular lipids. Mutations were identified in ATP-binding cassette transporter 1 (ABC1) in patients with genetic HDL deficiency (2–6), whose cells demonstrate impairment of the latter mechanism (7–10). This finding supported the idea that the assembly of HDL by apolipoprotein-cell interaction is a major source of HDL in the systemic circulation (1, 11). To date, the exact role of ABC1 in this reaction has not yet been fully understood, and its function in transmembrane cholesterol transport is proposed on the basis of the analogy to the function of other ABC transporters (12).

Since we reported HDL assembly by extracellular apolipoproteins with cellular lipids (13), it has been demonstrated that this reaction involves at least two independent components (1); one is a cellular interaction site for helical apolipoprotein to assemble HDL particles with cellular phospholipid (14–17) and the other is a signal-initiated specific intracellular cholesterol mobilization system for its incorporation into HDL (18–21). In an attempt to identify the mechanism in this specific intracellular cholesterol trafficking pathway, we found that an undifferentiated human monocytic leukemia cell line, THP-1, interacts with extracellular apolipoprotein A-I (apoA-I) and generates HDL with cellular phospholipid but no cholesterol. However, after the cells are differentiated by phorbol ester to the stage known to express various macrophage-like functions (22–25), cholesterol-rich HDL is generated by the cell-apolipoprotein interaction, indicating the induction of a reaction to incorporate cholesterol into the HDL. In the present article, we characterize the underlying mechanism of this phenomenon.

Abbreviations: ABC1, ATP-binding cassette transporter 1; ACAT, acyl-CoA:cholesterol acyltransferase; apo, apolipoprotein; BCA, bicinchoninic acid; BSA, bovine serum albumin; DRM, detergent-resisting membrane; FBS, fetal bovine serum; HDL, high density lipoprotein; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; RT-PCR, reverse transcription-polymerase chain reaction.

[1] To whom correspondence should be addressed.

MATERIALS AND METHODS

Cell culture

THP-1 cells were maintained in RPMI 1640 (Iwaki, Tokyo, Japan) containing 10% fetal bovine serum (FBS) (GIBCO-BRL, Gaithersburg, MD), or its lipoprotein-deficient serum fraction (d > 1.23 g/ml) (LPDS) if so mentioned, in a humidified atmosphere of 5% CO_2 and 95% air. For differentiation of THP-1 monocytes into macrophages, the cells were cultured in a six-well plate at a density of 3.0×10^6 cells/well in the presence of 3.2×10^{-7} M phorbol 12-myristate 13-acetate (PMA) (Wako, Osaka, Japan) for 72 h (23) except for the experiment to determine the effect of the length of the PMA treatment. The culture medium was replaced with RPMI 1640 supplemented with 0.2% bovine serum albumin (BSA) and incubated for a further 48 h. The undifferentiated cells were cultured in RPMI 1640 and 0.2% BSA for 48 h and seeded in a six-well plate at a cell density of 3.0×10^6/well.

Measurement of lipid release and intracellular lipid content

The differentiated and undifferentiated cells were cultured in the presence of lipid-free human apoA-I, apoA-II, or phosphatidylcholine-triolein microemulsion with a diameter of 25 nm (26), in 0.2% BSA-RPMI 1640 for 24 h. The lipid in the medium and the cells was extracted with chloroform–methanol 2:1 (v/v) and hexane–isopropanol 3:2 (v/v), respectively, for enzymatic determination of cholesterol (Kyowa Medics, Tokyo, Japan) and choline phospholipid (Wako). To measure the release of phosphatidylcholine and sphingomyelin, the cells were pulsed with [^{3}H]choline chloride (Amersham, Arlington Heights, IL) for the last 48 h before incubating with apoA-I (13). Radioactivity of choline phospholipid released was counted after the lipid in the medium was extracted and separated by thin-layer chromatography. The cells were dissolved in 0.1 N NaOH for protein determination by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL).

Density gradient ultracentrifugation

The apoA-I (20 μl/ml)-conditioned medium of the 12 wells (12 ml) was centrifuged at 1.64×10^5 *g* for 24 h at 4°C in a Hitachi (Tokyo, Japan) CP80b ultracentrifuge and the bottom 3.4 ml was collected. This fraction was overlaid on top of sucrose solutions of densities 1.31 g/ml (2.5 ml) and 1.15 g/ml (2.5 ml) in an ultracentrifuge tube for a Hitachi RP55T rotor. After centrifugation at 1.64×10^5 *g* for 48 h at 4°C, the solution was collected from the bottom into nine fractions. The contents of cholesterol and phospholipid as well as the density of each fraction were measured.

Measurement of intracellular cholesterol esterification and microsomal acyl-CoA:cholesterol acyltransferase activity

Incorporation of [^{14}C]oleic acid (0.45 μCi/ml; Amersham) into cholesteryl ester in the cells was determined (27). After incubation of the cells with apoA-I at 20 μg/ml, for 2 h, the cells were incubated with [^{14}C]oleic acid for an additional 1 h. The incorporation of radioactivity into the cholesteryl ester fraction was measured after lipid extraction and separation by thin-layer chromatography (method A). The microsomal fraction was prepared from THP-1 cells to measure its acyl-CoA:cholesterol acyltransferase (ACAT) activity (28). The cells were suspended in phosphate-buffered physiological saline and centrifuged at 650 *g* for 5 min to collect the pellet, to which 5 mM Tris-HCl (pH 8.5) was added and vortexed. The sample was chilled in ice and centrifuged at 650 *g* for 5 min, and the supernatant was centrifuged at 10,000 *g* for 30 min. The supernatant was collected and centrifuged at 105,000 *g* for 1 h to collect the pellet as the microsomal fraction. This fraction as 100 μg of protein was mixed with 250 nmol of cholesterol as cholesterol/phosphatidylcholine liposomes, and incubated in 0.1 M potassium phosphate buffer (pH 7.4) with [^{14}C]oleoyl-CoA (0.1 μCi/2 nmol) at 37°C for 5 min (method B). Alternatively, [^{14}C]cholesterol (1 μCi/50 nmol) was dissolved in 192 μl of 0.25% Triton WR-1339 and preincubated with 200 μg of microsomal protein in 8 μl at 37°C for 30 min, and the reaction was initiated by adding 30 nmol/4 μl of oleoyl-CoA at 37°C for 5 min (method C). Radioactivity in cholesteryl ester was counted after the lipid was extracted by chloroform–methanol 2:1. To evaluate an intracellular free cholesterol pool available for the ACAT reaction, the intracellular cholesterol esterification rate was standardized by the microsomal ACAT activity.

Antisense oligonucleotide treatment

The undifferentiated cells were suspended in 10% FBS-RPMI 1640 at a density of 5.2×10^7 cells/ml in the presence of a 20 mM concentration of the sense (5′-GCCAGCATGTCTGGGGG CAAATAC-3′), antisense (5′-GTATTTGCCCCCAGACATGCTG GC-3′), and scrambled (5′-TCGCACTTGTAGTTGGCCCGCA CA-3′) DNA for human caveolin-1, synthesized by ESPEC Oligo (Tsukuba, Japan). Control suspension contained cells without the oligonucleotides. Electroporation was carried out in a GenePulser (Bio-Rad, Hercules, CA) at 250 V, 1,100 μF, and then the cells were treated with PMA for differentiation. The apoA-I-mediated lipid release was measured as described above.

Preparation of cell membrane and detergent-resisting membrane fraction

The cells were harvested in 0.02 M Tris-buffered saline (pH 7.5) and pelleted by centrifugation at 600 *g* for 5 min. The cytosol and membrane fractions were separated by sonication of the cells in 20 mM phosphate buffer (pH 7.4) and centrifugation at 1.46×10^5 *g* as the supernatant and pellet, respectively. For the preparation of the detergent-resisting membrane (DRM) fraction (29), the cells were resuspended in 150 μl of 0.05 M boric acid buffer containing 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine (pH 7.2), and 3 ml of 0.02 M boric acid buffer containing 0.3 mM ethylenediaminetetraacetic acid (pH 10.2) was added. The suspension was strongly agitated for 10 min, mixed with 600 μl of 0.5 M boric acid (pH 10.2), and centrifuged at 600 *g* for 5 min. The supernatant was centrifuged at 12,500 *g* for 30 min, and the precipitate was sonicated in 0.02 M Tris-buffered saline (pH 7.5) containing the protease inhibitors and layered on the 35% sucrose solution. After centrifugation at 24,000 *g* for 1 h, the plasma membrane fraction at the interface was collected and sonicated in the 1% Triton X-100 and 8% sucrose solution, containing the protease inhibitors and 20 mM Tricine (pH 7.6), mixed with an equal volume of 60% sucrose, and centrifuged at 3.67×10^5 *g* for 2 h. The supernatant was diluted three times with 50 mM Tris buffer (pH 8.0) and the DRM fraction was precipitated by centrifugation at 3.67×10^5 *g* for 2 h. The protein content of each fraction was determined by the BCA method.

Western blotting of caveolin-1

Protein samples were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was blocked in 5% skim milk at 4°C overnight. After incubation with rabbit anti-caveolin-1 IgG (Santa Cruz Biotechnology, Santa Cruz, CA), the membrane was washed three times, 15 min each in 0.02 M Tris-buffered saline containing 0.05% Triton X-100 (pH 7.5), and incubated with anti-rabbit IgG conjugated with horseradish peroxidase (BioSource International, Camarillo, CA). The membrane

was washed and caveolin-1 was visualized by a chemiluminescence method.

RNA extraction and reverse transcription-polymerase chain reaction

Total RNA was extracted by the standard acid guanidinium thiocyanate-phenol-chloroform method. Briefly, the cells were lysed in the presence of phenol and guanidium and then RNA was recovered in the aqueous phase by adding chloroform and subsequent centrifugation. RNA was precipitated with isopropanol, and the pellet was washed with ethanol and dried. Total RNA content was determined by measuring the optical absorbance ratio at 260 to 280 nm after the sample was dissolved in diethylpyrocarbonate-treated water. First-strand cDNA was synthesized from the total RNA, 5 μg, and the cDNAs of caveolin-1 and ABC1 were amplified in a SuperScript preamplification system (GIBCO-BRL) and by polymerase chain reaction (PCR) for 26 cycles by using *Taq* polymerase (TaKaRa Shuzo, Osaka, Japan). Glyceraldehyde-3-phosphate dehydrogenase cDNA was also amplified as an intracellular standard. The cDNA was visualized by SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR) after 2% agarose gel electrophoresis. The quantitativeness of the assay was validated by the linearity with respect to the dose of total RNA and the number of the PCR cycles.

RESULTS

Figure 1 shows the effect of the differentiation of THP-1 cells by PMA on the removal of cellular cholesterol by apoA-I. The apoA-I-mediated cholesterol removal was induced by the PMA treatment of the cells in a time-dependent manner up to 72 h. ApoA-I-mediated HDL assembly was therefore analyzed in THP-1 cells after 72 h of PMA treatment (**Fig. 2**). When apoA-I was added to the culture

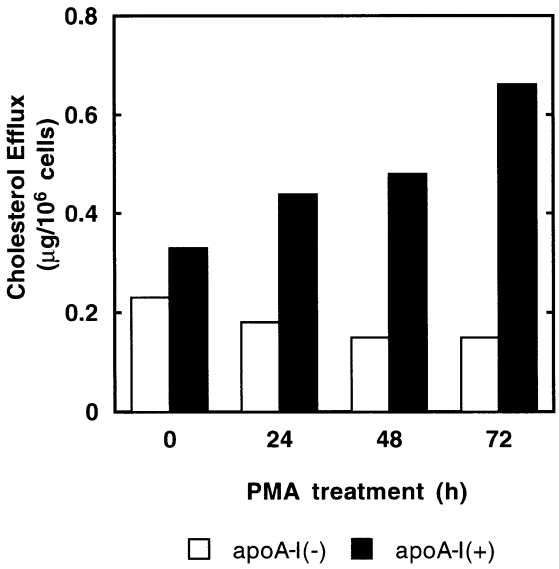

Fig. 1. Cholesterol removal by apoA-I from THP-1 cells after treatment with PMA for various lengths of time. The cells, 10^6/well, were treated with PMA for the indicated times, thoroughly washed, and then incubated with 10 μg of apoA-I. Cholesterol in the medium was measured after the incubation for 24 h. The data represent the average of two experimental points. The error between measurements was 7% at most.

medium, choline phospholipid was released into the medium from the cells in a dose-dependent manner in both the undifferentiated and differentiated stages, with a moderate increase in the differentiated condition. Cholesterol release by apoA-I was hardly detectable from the undifferentiated cells, whereas a substantial amount of cholesterol was released by apoA-I from differentiated cells. Further analysis of the choline phospholipid in the medium demonstrated that the release of phosphatidylcholine was hardly influenced by differentiation, whereas sphingomyelin release was increased in the same manner as cholesterol release. The HDL particles that appeared in the medium during the reaction were analyzed by density gradient ultracentrifugation. The HDL generated from undifferentiated cells contained a small amount of cholesterol whereas the HDL from differentiated cells was rich in cholesterol. The increase in HDL cholesterol was more than 15-fold in terms of the ratio to phospholipid.

ApoA-I-mediated cholesterol removal was analyzed in relation to the levels of cellular cholesterol. Differentiated cells contained a slightly higher amount of free and esterified cholesterol than undifferentiated cells, and it seems an extracellular supply of cholesterol, as the LPDS-conditioned cells had less cholesterol than the FBS-conditioned cells (**Fig. 3**, bottom). Cellular esterified cholesterol was decreased by apoA-I in differentiated cells, whereas cellular cholesterol did not change in undifferentiated cells, reflecting the lack of its removal by apoA-I. The apoA-I-mediated cholesterol removal from differentiated cells increased when cell cholesterol increased (Fig. 3, top left). When cholesterol was further loaded into differentiated cells by incubating them with acetylated low density lipoprotein (LDL) to the extent that cellular esterified cholesterol was 17.3 ± 1.1 μg/mg cell protein, the apoA-I-mediated release was up to 10.6 ± 0.4 μg/mg cell protein (data not shown). Because phospholipid removal did not change by cell cholesterol level (Fig. 3, top right), the HDL generated by this reaction was enriched in cholesterol as the cellular cholesterol increased in the differentiated THP-1 cells. These data were consistent with the finding by Bielicki, McCall, and Forte (30). Cholesterol release from undifferentiated cells was again shown to be extremely low, whereas phospholipid release was essentially the same as in differentiated cells (Fig. 3, top). Cholesterol loading of undifferentiated cells via LDL resulted only in a slight increase in cellular cholesteryl ester. The apoA-I-mediated cholesterol release hardly increased and the reciprocal decrease in cellular cholesterol ester by apoA-I did not reach a detectable level (**Fig. 4**).

A cellular cholesterol compartment accessible by ACAT was probed (**Table 1**). Intracellular incorporation of radiolabeled oleic acid into cholesteryl ester was measured by using whole cells (Table 1, column A). The enzyme activity of the microsomes was measured separately by two independent methods because it can be altered by differentiation (Table 1, columns B and C). The compartment indicator (A) was then standardized by the enzyme activity (B and C). The enzyme activity increased twice by the PMA treatment and the ACAT-available cholesterol compart-

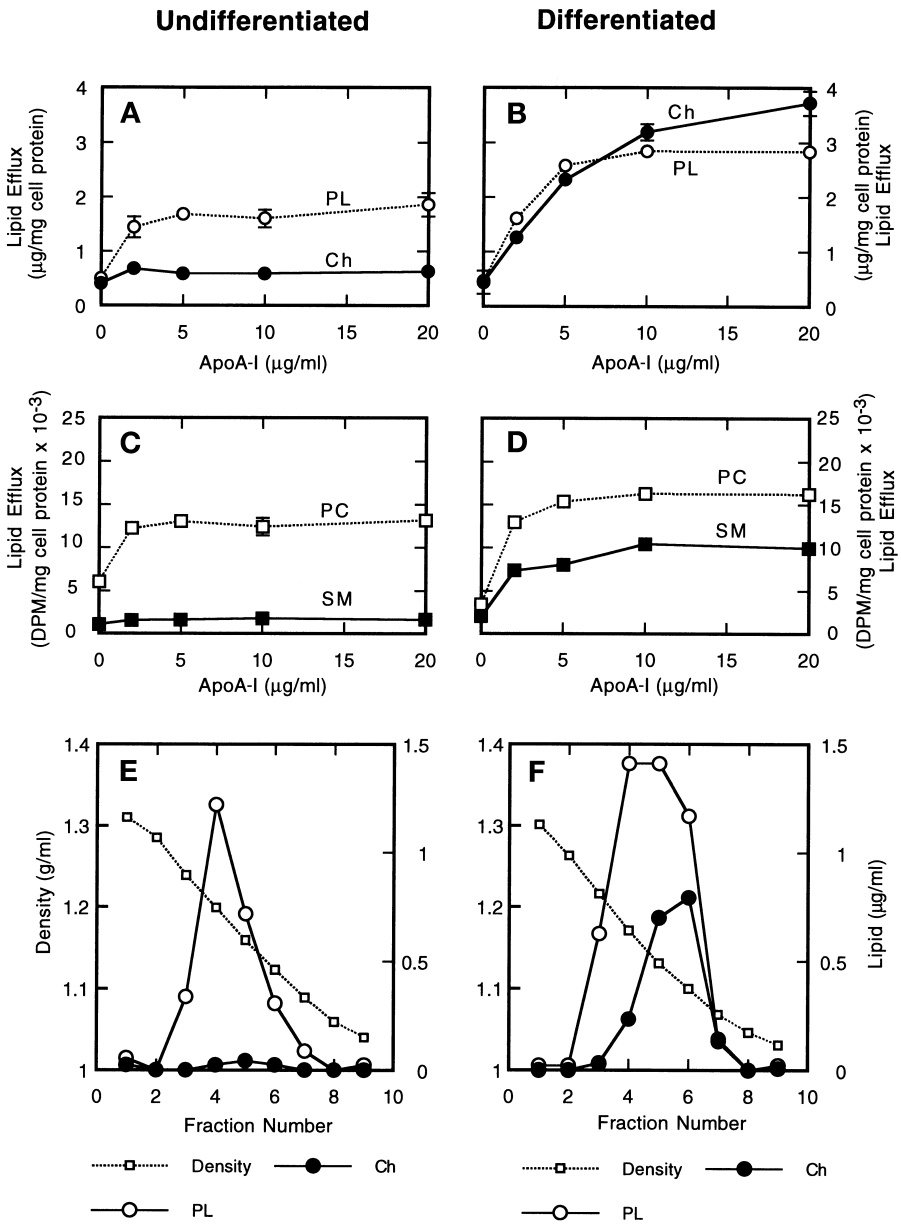

Fig. 2. Release of lipids by apoA-I from THP-1 cells. (A and B) Release of choline-phospholipid (PL) and cholesterol (Ch) mass into 1 ml of medium from undifferentiated and differentiated THP-1 cells (575 ± 7 and 639 ± 19 μg of cell protein per well, respectively) in the presence of apoA-I. (C and D) Release of choline-labeled phosphatidylcholine (PC) and sphingomyelin (SM) from undifferentiated and differentiated THP-1 cells (534 ± 13 and 555 ± 7 μg/well). (E and F) Density gradient ultracentrifugation analysis of the medium of undifferentiated and differentiated THP-1 cells (total 7.7 and 8.1 mg of cell protein, respectively) after incubation with apoA-I. The data represent the average ± SE of three experimental points for (A–D). Error bars are hidden by symbols for some data points. The data in (E) and (F) are the results of the average of duplicate assays for a single data point. DPM, radioactivity as decay per minute.

ment remained the same after the standardization (A/B and A/C). This compartment decreased with the release of cholesterol by apoA-I only in differentiated cells, whereas the undifferentiated cells did not respond to apoA-I, reflecting the lack of cholesterol incorporation into the HDL assembled.

Figure 5 shows cholesterol release by apoA-II and by lipid microemulsion. ApoA-II-mediated cholesterol release was also induced by cellular differentiation by PMA. In contrast, cholesterol release induced by the lipid microemulsion by nonspecific diffusion was not influenced by differentiation. The finding with apoA-II was consistent with the previous view that the reaction to generate HDL from cellular lipids is not highly specific to apoA-I, but is mediated by many other helical apolipoproteins (31, 32). The induction of cholesterol release by cellular differentiation is limited to the apolipoprotein-mediated reaction, and is not extended

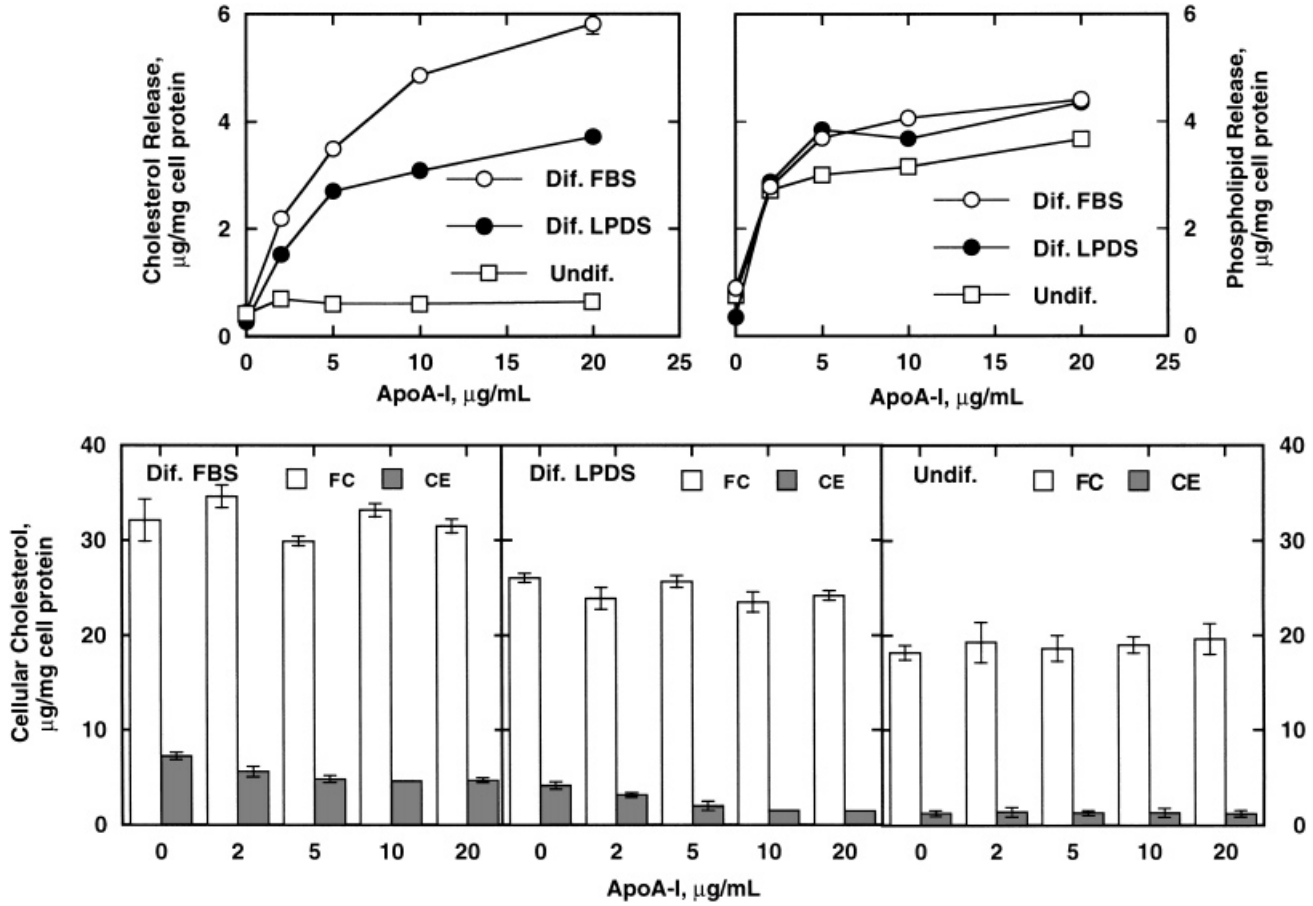

Fig. 3. Intracellular cholesterol of THP-1 cells after incubation with apoA-I (lower three panels) in relation to cholesterol and phospholipid release (upper two panels). Cell protein was 541 ± 24, 500 ± 5, and 549 ± 6 μg/well for the undifferentiated (Undif.), differentiated in FBS (Dif. FBS), and differentiated in LPDS (Dif. LPDS) cells, respectively. Cholesterol and choline phospholipid in the medium were measured as described in text. The data represent the average of two measurements, whose error was 7% at most. Cellular total and free cholesterol (FC) were also measured as described in text and the difference was calculated as the esterified cholesterol (CE). The data represent the means ± SE for triplicate measurements.

to nonspecific diffusion of cholesterol from the cellular surface.

Figure 6 demonstrates the induction of gene expression by cellular differentiation. The message levels of ABC1 and caveolin-1 are shown as the products of reverse transcription (RT)-PCR. Both messages are markedly increased in the cells differentiated by PMA. **Table 2** represents the amounts of ABC1 and caveolin-1 message. Caveolin-1 protein also markedly increased after cell differentiation by PMA, shown by immunoblotting (Fig. 6B). When expression was induced, caveolin-1 was rich in the DRM.

To differentiate the role of caveolin-1 from that of ABC1 in apoA-I-mediated cholesterol release, the cells were treated with an antisense DNA against caveolin-1 (**Fig. 7**). Synthesis of caveolin-1 protein was significantly decreased as demonstrated by immunoblotting. Whereas the choline phospholipid release by apoA-I was not influenced by the antisense treatment, the cholesterol release from the antisense-treated cells was selectively reduced. Release of cholesterol into the medium without apoA-I was 0.18 ± 0.05 μg/mg cell protein from the sense DNA-treated cells and 0.20 ± 0.06 μg/mg cell protein from the antisense DNA-treated cells, as the means ± SE of the data from five independent series of experiments. Accordingly, the cholesterol-to-phospholipid ratio in the apoA-I-mediated lipid release markedly decreased from that of the antisense-treated cells, demonstrating the production of cholesterol-poor HDL in the medium. The apoA-I-conditioned medium of each treatment group was analyzed by density gradient ultracentrifugation (**Fig. 8**). The medium of each treatment group contained HDL generated by the apoA-I-cell interaction, and the HDL generated with the antisense DNA-treated cells was less rich in cholesterol. Cellular cholesterol change is shown in **Fig. 9**. Unesterified and esterified cholesterol content was the same in cells treated with sense, antisense, and scrambled DNAs, and no significant change in unesterified cholesterol was induced by apoA-I in any of these cells. On the other hand, esterified cholesterol decreased by apoA-I in the cells treated with sense and scrambled DNA but not in those treated with antisense DNA. **Figure 10** demonstrates correlation of apoA-I-mediated cell cholesterol release with caveolin-1 protein expression level by antisense DNA treatment, with respect to the relative decrease from sense DNA-treated cells. The decrease in cho-

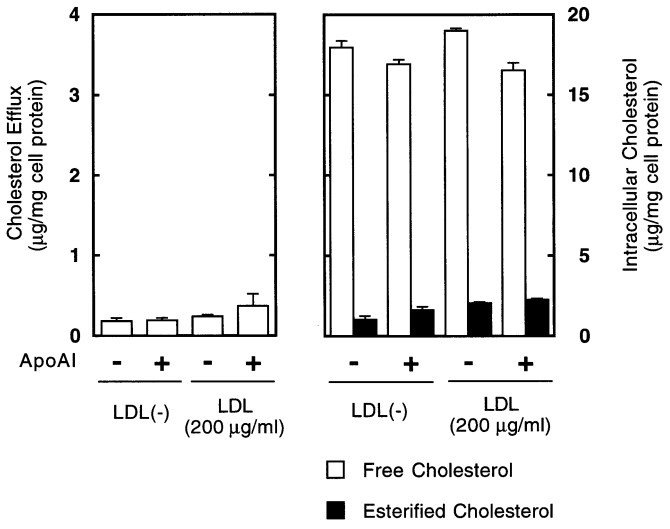

Fig. 4. The effect of cholesterol loading of undifferentiated cells on apoA-I-mediated cholesterol removal and cellular cholesterol. The cells were incubated with LDL (200 μg/ml medium) for 24 h, washed, and then incubated with apoA-I (20 μg/ml) for 24 h. Total cholesterol in the medium and total and free cholesterol in the cell were measured as described in text. The esterified cholesterol was calculated as the difference between total and free cholesterol. Cell protein was 419.9 ± 7.8 μg/well of the cholesterol-unloaded cells and 363.1 ± 14.1 μg/well of the cholesterol-loaded cells.

lesterol release was linearly correlated with the level of caveolin-1 protein expression.

DISCUSSION

We have demonstrated here that the differentiation of THP-1 cells by phorbol ester resulted in the selective cholesterol enrichment of the HDL that is generated by the apolipoprotein-cell interaction, being independent of the diffusion-mediated cellular cholesterol efflux. Two proteins known to be functional in intracellular cholesterol transport are induced in the differentiation: ABC1, which has been shown to be mutated in genetic HDL deficiencies (2–6), and caveolin-1, which reportedly accelerates cholesterol transport to the plasma membrane (33, 34). The expression of caveolin-1 was suppressed by antisense DNA treatment of differentiated cells, and cholesterol incorporation was selectively decreased in the HDL generated by the apolipoprotein-cell interaction.

PMA treatment did not influence cellular cholesterol efflux by a nonspecific diffusion-mediated mechanism. Therefore, induction of cellular cholesterol release by cell differentiation was restricted to the apolipoprotein-mediated pathway and was independent of the size at the cellular surface of the cholesterol pool that undergoes nonspecific diffusion through the aqueous phase to "acceptors," such as extracellular lipid particles. Thus, the effect of the PMA treatment cannot be attributed to a general increase in surface cholesterol, and is perhaps related to involvement of specific intracellular cholesterol trafficking for the apolipoprotein-mediated reaction.

Apolipoprotein-mediated cellular cholesterol removal is a cellular cholesterol release mechanism distinct from the nonspecific release mediated by diffusion (1). This reaction assembles HDL particles with apolipoprotein and cellular lipid and requires a cellular interaction site for apolipoprotein (14–17) and an intracellular cholesterol transport system specific for incorporation of cholesterol into HDL (18–21). The reaction has also been implicated as a major source of plasma HDL, by the impairment of this reaction in Tangier disease, the genetic HDL deficiency (7–10), and by the inhibition of this reaction by the HDL-lowering reagent probucol (16). This speculation was further confirmed by the finding that the primary gene defect in Tangier disease is in the membrane transporter protein ABC1 (2–6). Because the previous reports agree that Tangier cells lack the removal of both cholesterol and phospholipid by apolipoprotein and hence lack the assembly of HDL (7–10), it is likely that ABC1 is directly involved in the interaction of the cells and apolipoprotein. Cholesterol removed by this pathway originates in the intracellular compartment that is readily accessible by ACAT (19, 20, 27), implicating the involvement of a cho-

TABLE 1. Incorporation of ^{14}C[oleic] acid into cholesteryl ester and ACAT activity of the microsomal fraction in undifferentiated and differentiated THP-1 cells[a]

	A	B	C	A/B	A/C
Undifferentiated					
ApoA-I (−)	3,675 ± 301	34.7 ± 0.6	14.2 ± 1.5	105.9 ± 8.6	258.8 ± 21.1
ApoA-I (+)	3,505 ± 34	—	—	101.0 ± 1.0	246.8 ± 2.4
Differentiated					
ApoA-I (−)	9,273 ± 636	89.5 ± 0.8	31.9 ± 0.8	103.6 ± 7.1	290.7 ± 19.9
ApoA-I (+)	7,088 ± 213	—	—	79.2 ± 2.4[b]	222.1 ± 6.7[c]

[a] The cells were incubated with apoA-I (20 μg/ml) for 2 h at 37°C and then [^{14}C]oleic acid was added to the medium for the incubation for an additional 1 h to measure its incorporation into cholesteryl ester (A) (dpm/mg cell protein). The ACAT activity in the cells was estimated with the microsomal fraction as the cholesterol esterification rate by measuring incorporation of [^{14}C]oleyl CoA into cholesteryl ester (B) and by measuring esterification of [^{14}C]cholesterol (C) (pmol/min/mg microsomal protein) according to the method described in text. The values represent means ± SE of the triplicated assay. The A values are standardized by the B and C values in order to estimate the intracellular cholesterol compartment available for the ACAT reaction (A/B and A/C).

[b] $P < 0.01$ from other A/B values.

[c] $P < 0.01$ from other A/C values.

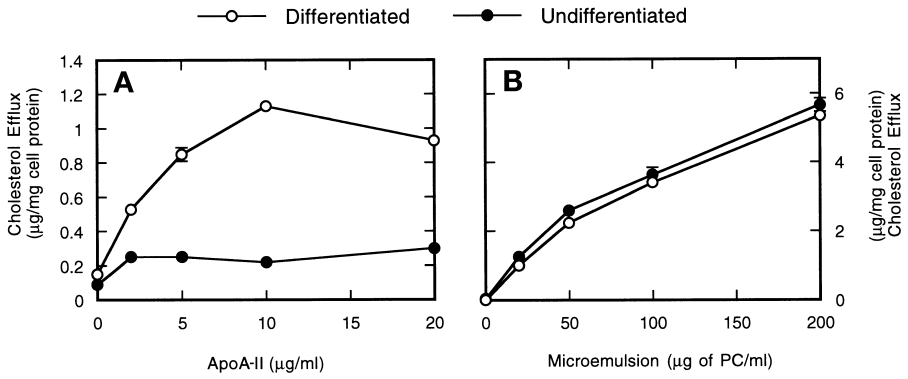

Fig. 5. Release of cholesterol from THP-1 cells by apoA-II (A) and by lipid microemulsion (B). The undifferentiated (closed circles) and differentiated (open circles) cells were incubated with human apoA-II or a lipid microemulsion of phosphatidylcholine (PC) and triolein, with an average diameter of 25 nm, and cholesterol mass release into the medium was determined. Cell protein: apoA-II, PMA(−) 613 ± 12 μg/well and PMA(+) 531 ± 5 μg/well; microemulsion, PMA(−) 660 ± 10 μg/well and PMA(+) 465 ± 8 μg/well. The data represent the average ± SE of three experimental points. Error bars are hidden by symbols for some data points.

lesterol trafficking pathway before it reaches the plasma membrane domain, where ABC1 potentially functions.

The data presented here have shown that HDL is assembled without cholesterol in undifferentiated THP-1 cells on interaction with apolipoproteins. The HDL was enriched in cholesterol by the PMA treatment of the cells, without a drastic increase in the production of HDL itself. These results indicate that the interaction of apolipoprotein with the cells to generate HDL takes place independently of the removal of cellular cholesterol by this reaction. Dissociation of these two steps in apolipoprotein-mediated HDL assembly was in fact previously demonstrated by modula-

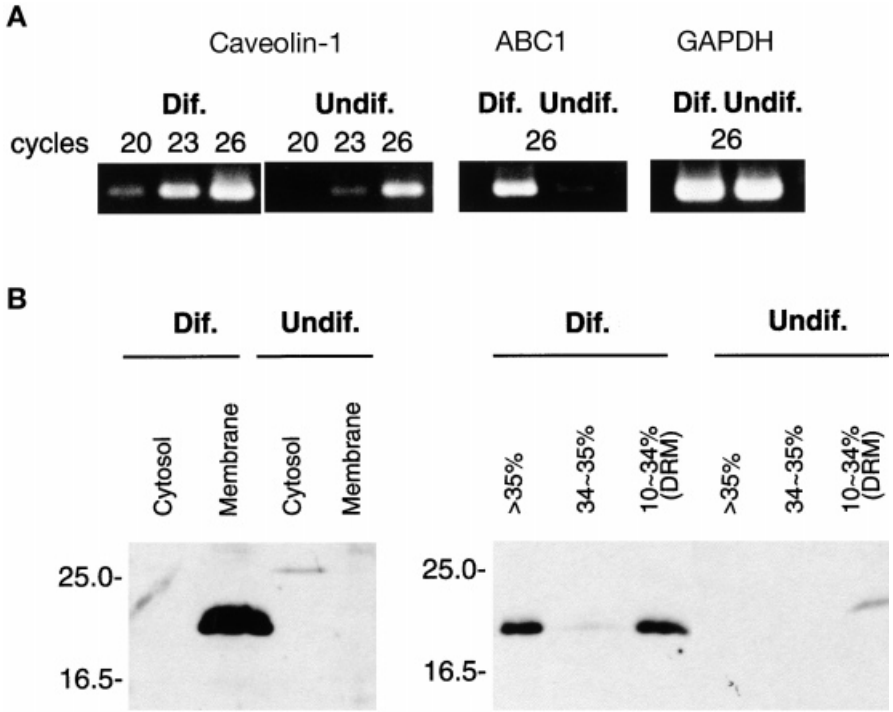

Fig. 6. Induction of caveolin-1 and ABC1 in THP-1 cells with differentiation by PMA. (A) RT-PCR amplification products for the message of GAPDH, caveolin-1, and ABC1. (B) Western blotting analysis of caveolin-1, in the cytosol, membrane, and DRM fractions of THP-1 cells, before and after differentiation by PMA. One-tenth of the whole preparation was applied for "cytosol" and "membrane" (approximately 30 μg of protein), and 10 μg of protein for the pellet of each centrifugation step of the DRM preparation; >35%, the fraction precipitated in 35% sucrose; 34–35%, the precipitated fraction in 34% sucrose after treatment with 1% Triton X-100 of the interface fraction in 35% sucrose; 10–34%, the precipitated fraction in 10% sucrose of the Triton X-100-treated supernatant in 34% sucrose.

TABLE 2. Expression of ABC1 and caveolin-1 message in THP-1 cells by differentiation with PMA[a]

	PMA(−)	PMA(+)
ABC1	0.039 ± 0.006	0.758 ± 0.009
Caveolin-1	0.156 ± 0.003	0.659 ± 0.023

[a] Density of the electrophoresis band of the RT-PCR products was quantitated by digital scanning with a Epson image scanner GT-9500 ART and standardized for GAPDH message. The values represent the means and SE of three specimens.

tion of protein kinase C in rat vascular smooth muscle cells (19) and murine peritoneal macrophages (20), and by alteration of intracellular cholesterol levels in differentiated THP-1 cells (30).

It is interesting that expression of ABC1 message with differentiation did not parallel the production of HDL, but was apparently associated only with cholesterol incorporation into HDL. At least a few ABC1 mutations may disrupt the removal of cellular phospholipid by apoA-I (7–10), so that this protein is likely to be involved in the interaction of apolipoprotein with cell membrane. The present data may therefore be interpreted as suggesting that the low level of ABC1 before differentiation is already adequate to mediate the apolipoprotein-cell interaction, or there may be an alternative mechanism to mediate this interaction to generate HDL. In the former case, ABC is already functional at the initial low expression level, and the massive induction of cholesterol incorporation into the HDL assembly process can be caused by some other factor(s). Otherwise, the level of ABC1 required for the apolipoprotein interaction is different from that for transmembrane cholesterol export.

Expression of caveolin-1 was also highly induced by PMA treatment, in agreement with another report (35). This is also potentially associated with the massive increase in cholesterol export because this protein has been thought to function in intracellular cholesterol transport. In support of this view, suppression of increased caveolin-1 expression by antisense DNA led to selective reduction of cholesterol incorporation into HDL assembled by the apoA-I-cell interaction and resulted in production of cholesterol-poor HDL. Thus, caveolin-1 was shown to play an independent regulatory role in the provision of cholesterol for the HDL assembly mechanism by apolipoprotein with the cellular lipids. The results were largely consistent with a previous report (36) that the message level of caveolin-1 was associated with cellular cholesterol release to plasma, with respect to the increase in relation to the cellular free cholesterol level and to the decrease by antisense DNA treatment. The present article demonstrates that the involvement of caveolin-1 in cellular cholesterol release is specific to the pathway associated with apolipoprotein-mediated HDL assembly. It is likely to be a tandem step to ABC1-mediated cholesterol export at the plasma membrane because the assembly of HDL with phospholipid was least influenced by the induction and suppression of caveolin-1 expression. It is interesting to note the upregulation of caveolin-1 in multidrug-resistant cancer cells, because this cell stage is closely related to the function

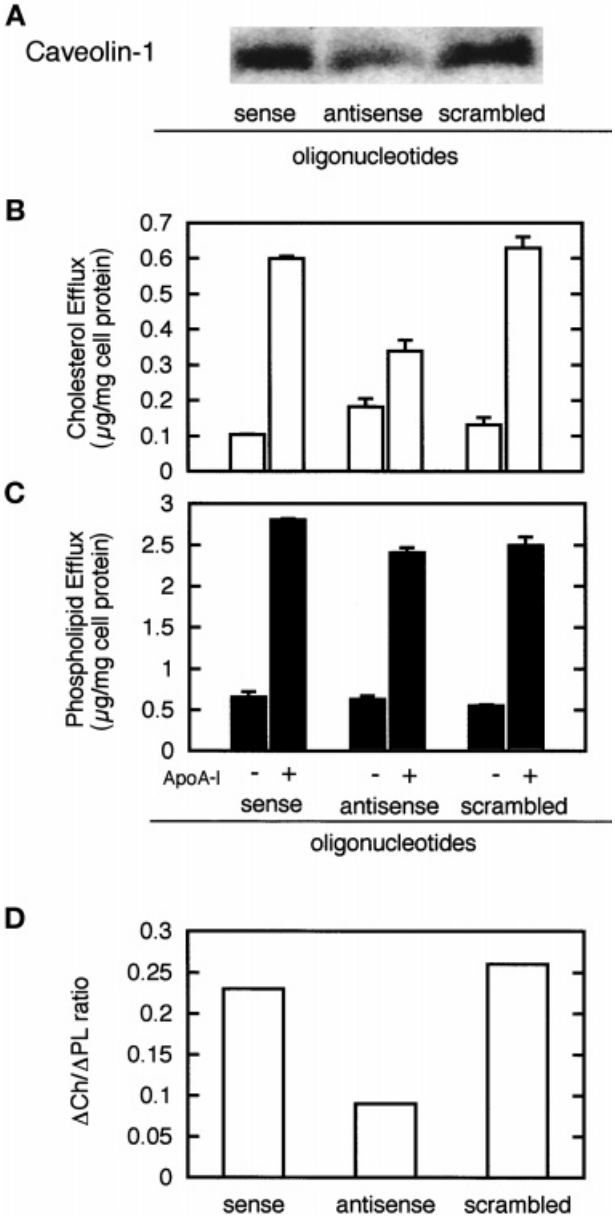

Fig. 7. The effect of caveolin-1 antisense DNA on apoA-I-mediated lipid release from differentiated THP-1 cells. (A) Western blotting analysis for caveolin-1, using 30 μg of membrane protein of cells treated with sense, antisense, and scrambled DNA. (B) Cholesterol (Ch) release from treated cells in the absence and presence of apoA-I (cell protein, 593 ± 17, 547 ± 14, and 605 ± 17 μg/well, respectively). (C) Choline-phospholipid (PL) release from treated cells in the absence and presence of apoA-I, 20 μg/ml (cell protein, 580 ± 9, 572 ± 10, and 623 ± 14 μg/well, respectively). (D) Mass ratio of Ch to PL in apoA-I-mediated lipid released into the medium by the treated cells. The data in (B) and (C) represent the average ± SE of three experimental points. The data in (D) were calculated from the average data values in the (B) and (C).

of ABCs (37). Also, it should be stated that the present experiments were carried out mostly in cells into which cholesterol was not loaded, where ABC1 is not fully expressed (38). The cholesterol-loaded condition should therefore be tested more extensively in future experiments.

Caveolin-1 is understood to play an important role in the

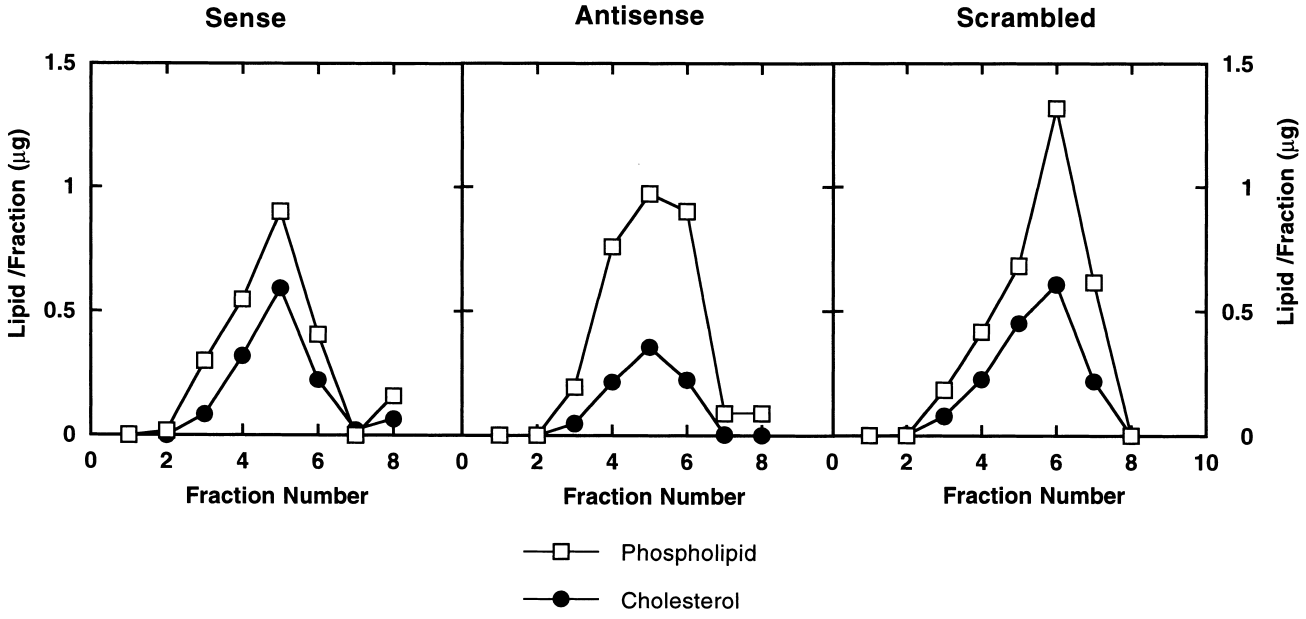

Fig. 8. Density gradient analysis of medium after the incubation with apoA-I of cells treated with sense, antisense, and scrambled DNAs. The experimental conditions were similar to those described in Fig. 7. The samples were fractionated from the bottom to the top. Choline-phospholipid and cholesterol were measured for each density fraction after centrifugation. The data points represent single assay data.

regulation of intracellular cholesterol homeostasis, although its precise function has not yet been fully elucidated. It is found in association with the cholesterol/sphingomyelin-rich domain of the plasma membrane, which is often identified as a membrane fraction DRM (39). Expression of caveolin-1 is closely related to cellular cholesterol levels (36, 40), and its association with membrane is regulated by the membrane cholesterol level (41). It has also been reported that caveolin-1 enhances cholesterol transport from the intracellular compartment to the plasma membrane (33) by forming a complex with a chaperone protein(s) in the cytosol (34). Furthermore, cholesterol in DRM seems to be selectively involved in the efflux to HDL (42) that includes both nonspecific and apolipoprotein-mediated mechanisms. Our finding indicated that cholesterol used by the apolipoprotein-mediated HDL assembly is strongly associated with sphingomyelin in DRM (43). In THP-1 cells, selective uptake of HDL cholesteryl ester by scavenger receptor

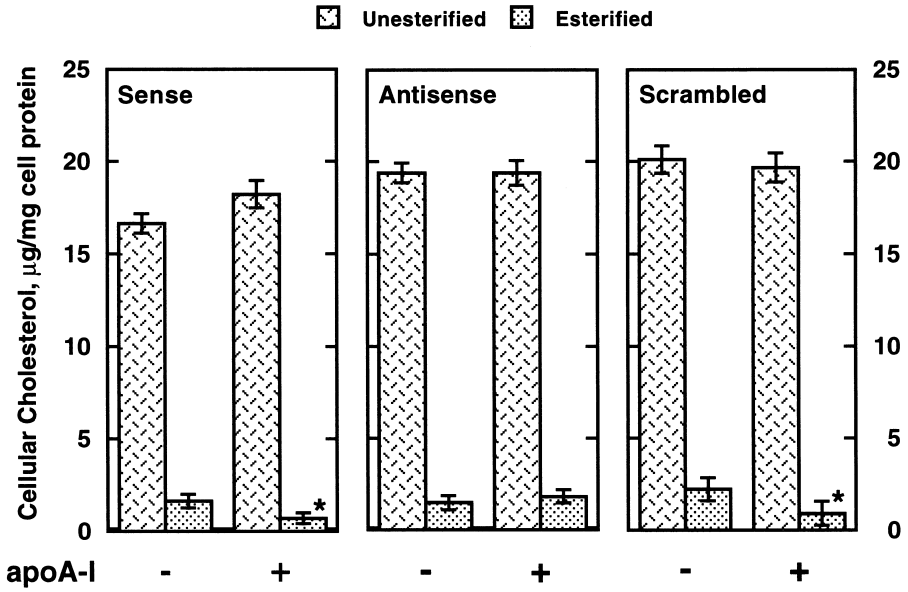

Fig. 9. Change in cellular cholesterol subsequent to apoA-I-induced HDL assembly in THP-1 cells treated with sense, antisense, and scrambled DNAs. The experimental conditions were similar to those described in Fig. 7, and the data represent the means ± SE of triplicate assays. Asterisks indicate a significant change ($P < 0.05$) by apoA-I.

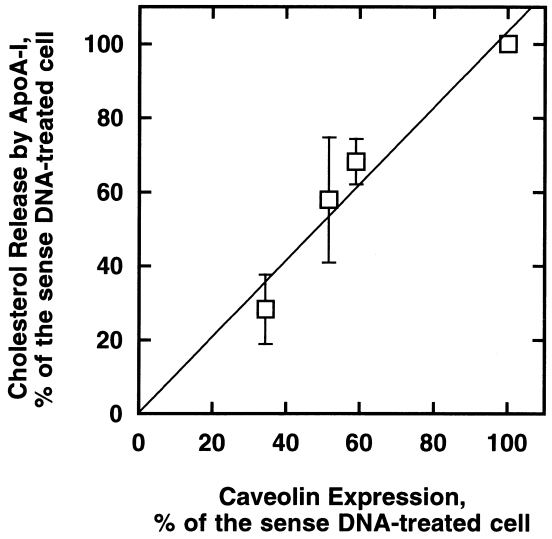

Fig. 10. Decrease in apoA-I-mediated cholesterol release (by 20 μg/ml medium) and caveolin-1 expression quantitated by Wetern blotting. The parameters with the antisense DNA-treated cells are expressed as the percentage value relative to those with sense DNA-treated cells (100%) in the pair-matched experiment. The data represent means ± SE of three independent series of experiments. The straight line represents the least-squares linear regression, including the 100% data point.

B-1 increased with PMA-mediated differentiation, and this can be attributed to the expression of caveolin-1 (35).

The underlying mechanism by which caveolin-1 regulates a specific intracellular cholesterol trafficking pathway to apolipoprotein-mediated HDL assembly is still unknown. Suppression of cholesterol incorporation into HDL by caveolin-1 antisense DNA suggests that this protein is required for the trafficking. It should be determined whether the increase in caveolin-1 expression is a primary cause of the increase in cholesterol incorporation into HDL when THP-1 cells are differentiated.

A small increase in intracellular cholesteryl ester was observed with PMA treatment. This increase can be caused partially by the uptake of denatured LDL by induction of scavenger receptor A (23) or by an increase in SRB1-mediated HDL-cholesteryl ester uptake (35) from FBS because it was lower when the cells were conditioned by LPDS instead of FBS, in agreement with the previous report that intracellular cholesterol levels are related to the cholesterol enrichment of HDL generated with differentiated cells (30). On the other hand, the increase in cellular cholesterol by LDL loading of undifferentiated cells did not cause massive cholesterol enrichment of HDL. It is not known clearly what intracellular cholesterol or cholesteryl ester compartment is used for apolipoprotein-mediated HDL assembly, except for the limited observation of the preferred use of the compartment readily accessible by ACAT. The data in Table 1 at least demonstrated that the ACAT-available intracellular cholesterol compartment does not seem to change with PMA treatment. In addition, loading cholesterol into undifferentiated cells does not lead to such a marked increase in cholesterol incorporation into HDL as is observed with differentiated cells. Therefore, it is likely that induction of caveolin-1 expression can be a primary cause of cholesterol enrichment of HDL by PMA-induced cell differentiation, by intracellular cholesterol mobilization from the ACAT-available compartment to that for apolipoprotein-mediated HDL assembly.

This work has been supported in part by grants-in-aids from the Ministry of Science, Education and Culture, and from the Ministry of Health and Welfare.

Manuscript received 3 February 2000, in revised form 21 April 2000, in re-revised form 19 June 2000, and in re-re-revised form 19 July 2000.

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