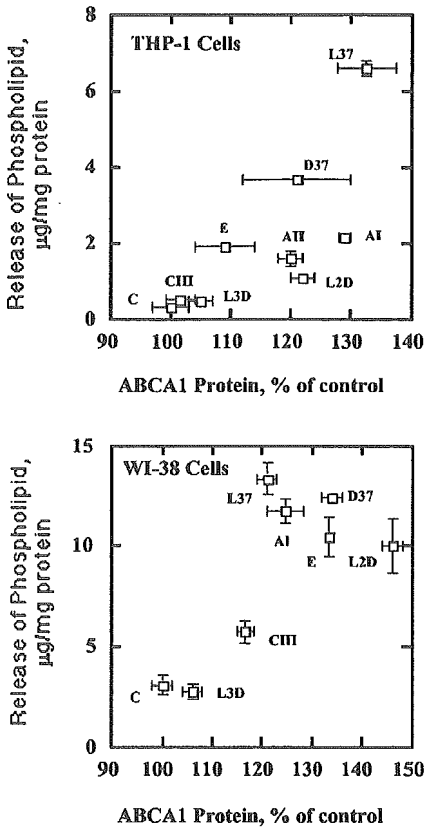

FIG. 8. **Relationship of choline-phospholipid release by the apolipoproteins or by the synthetic peptides to ABCA1 protein level, for THP-1 cells and WI-38 cells.** The data used in this figure and the data in Figs. 1 and 2 are plotted.

lipids release and ABCA1 protein levels. A direct positive correlation was observed between ABCA1 phosphorylation and ABCA1 protein level (Fig. 7). Finally, phospholipid release increased along with ABCA1 protein level, shown in Fig. 8.

We thus conclude that structural requirement of apolipoprotein for removal of lipid to generate HDL is the presence of a stable type A amphiphilic helical segment (13) and that this structural motif is also strongly associated with the potential of a protein to promote the phosphorylation and stabilization of ABCA1.

There was, however, some interesting differences in the ability of the various peptides to promote lipid efflux from THP-1 cells and WI-38 cells (Figs. 1 and 2). The L37pA and D37pA peptides were more effective in lipid release in THP1 cells than apolipoproteins and the L2D37pA peptide. ApoA-I and L2D37pA peptide were previously shown to be completely dependent upon ABCA1 for lipid release, whereas L37pA and D37pA, because of their higher lipid affinity, are also able to promote lipid release without ABCA1 (13). It is, therefore, possible a lower level of ABCA1 activity in THP1 cells compared with WI-38 cells could account for this difference. Alternatively, a difference in the lipid composition between THP-1 cells and WI-38 cells could also account for a greater amount of ABCA1-independent lipid efflux from the THP1 cells for the L37pA and D37pA peptides. Previously, it was shown in HeLa cells transfected with ABCA1 (13) that there was not a stereoselective interaction with synthetic amphiphilic helical peptides in the lipid release process, which is consistent with the equal amounts of lipid release observed for the L and D stereoisomer forms of the 37pA peptide observed in WI-38 cells. It was consistently observed in this study, however, that the D37pA peptide was not as effective as the L37pA peptide for lipid release in THP1 cells. This suggests that there must be some sort of chiral interaction of the peptides with the THP1 cells, which could be with some other auxiliary protein besides ABCA1 to promote the cell interaction with the L37pA peptide and or in some other way to promote lipid release. In regard to the effect of the peptides on ABCA1 phosphorylation and ABCA1 protein stabilization, the two cell lines yielded similar results. Those peptides and apolipoprotiens that stimulated phospholipid efflux also promoted the phosphorylation of ABCA1 and its stabilization against degradation (Figs. 3 and 4). Because the 37pA peptides do not share any primary amino acid homology with any apolipoprotein (13), but do have type A amphiphilic helices mimicking apolipoproteins, ABCA1 phosphorylation and stabilization is unlikely to be the result of a specific interaction of the peptides or apolipoproteins with the ABCA1 transporter. This view is further supported by the observation that the L and D stereoisomer forms of the peptides were equally effective in promoting the phosphorylation and stabilization of ABCA1 (Figs. 3 and 4). A positive correlation was observed between phospholipid release and ABCA1 phosphorylation and stabilization (Figs. 6–8), which as proposed previously (10) suggests that the release of phospholipid from cells provides the signal for ABCA1 phosphorylation and stabilization. Such a mechanism would provide an alternative means besides gene regulation for regulating the amount of functional ABCA1 transporter present in cells and for coupling the amount of ABCA1 in cells with the availability of HDL-apolipoproteins in extracellular fluid.

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Probucol Inactivates ABCA1 in the Plasma Membrane with Respect to Its Mediation of Apolipoprotein Binding and High Density Lipoprotein Assembly and to Its Proteolytic Degradation*

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Probucol has been shown to inhibit the release of cellular lipid by helical apolipoprotein and thereby to reduce plasma high density lipoprotein. We attempted to explore the underlying mechanism for this effect in human fibroblast WI-38. Probucol inhibited the apoA-I-mediated cellular lipid release and binding of apoA-I to the cells in a dose-dependent manner. It did not influence cellular uptake of low density lipoprotein, transport of cholesterol to the cell surface whether *de novo* synthesized or delivered as low density lipoprotein, and overall cellular content of cholesterol, although biosynthesis of lipids from acetate was somewhat increased. Probucol did not affect the mRNA level of ABCA1, and ABCA1 was recovered along with marker proteins for plasma membrane regardless of the presence of probucol. However, the protein level of ABCA1 increased, and the rate of its decay in the presence of cycloheximide was slower in the probucol-treated cells. ABCA1 in the probucol-treated cells was resistant to digestion by calpain but not by trypsin. We concluded that probucol inactivates ABCA1 in the plasma membrane with respect to its function in mediating binding of and lipid release by apolipoprotein and with respect to proteolytic degradation by calpain.

Cholesterol is an essential molecule for animal cells to maintain and regulate function and structure of the biomembrane. It is synthesized in most somatic cells, whereas its catabolic site is limited to the liver and to the steroidogenic cells except for partial hydroxylation in some somatic cells. Accordingly, cholesterol is removed from the cells and transported to the liver for its conversion to bile acids, and this is one of the essential events in cholesterol homeostasis for the body and for the cells (1). High density lipoprotein (HDL)[1] is believed to play a central role in this system, and this is thought to be one of the antiatherogenic characteristics of HDL. This reaction takes place through at least two distinct mechanisms: 1) physicochemical release of cholesterol from the cell surface, which is driven by cholesterol esterification on HDL, and 2) the apolipoprotein-mediated pathway to remove cellular cholesterol and phospholipid to generate new HDL particles (2). HDL thus plays a central role in both mechanisms.

Apolipoprotein-dependent cellular cholesterol release is absent in fibroblasts from patients with Tangier disease (3, 4), and mutations in the gene encoding the ATP-binding cassette transporter A1 (ABCA1) are the underlying cause of this disease (5–9). On the other hand, *in vitro* overexpression of functional ABCA1 in the cells (10, 11) and induction of ABCA1 expression by cyclic AMP analogues (12, 13) or by the ligands for the liver X receptor or retinoid X receptor (14, 15) enhanced the release of cellular cholesterol and phospholipid by apolipoprotein. The transgenic mice for ABCA1 had a significant increase in plasma HDL (16, 17). These results indicate that this protein is a regulating factor for the plasma HDL level through generation of HDL by the apolipoprotein-cell interaction.

Probucol has been clinically used as an antiatherogenic compound, not only because of its lipid-lowering effect but also because of the hypothesis that its antioxidative nature prevents atherogenic oxidative modification of low density lipoprotein (LDL) shown by *in vitro* (18, 19) and *in vivo* models (20, 21). However, probucol substantially reduces plasma HDL (22). We reported that probucol causes dramatic selective inhibition of the apolipoprotein-mediated cellular lipid release and its binding to cells (23) to cause reduction of HDL (24), which is analogous to the finding with Tangier disease (3, 4). Thus, this is another piece of evidence that the apolipoprotein-cell interaction to generate HDL is a major source of plasma HDL. Probucol can therefore be considered an inhibitor of the function of ABCA1.

Cellular ABCA1 undergoes both transcriptional and post-transcriptional regulations (25). Transcriptional regulation of ABCA1 is carried out by oxysterol through the liver X receptor/retinoid X receptor system, which seems relevant to the function of ABCA1 to expel an excess amount of cell cholesterol (25). Up-regulation of ABCA1 is also mediated by other factors such as cyclic AMP (13), of which the exact mechanism is unknown. On the other hand, stabilization of ABCA1 can be an alternative mechanism to regulate ABCA1 activity, such as protection of ABCA1 protein by helical apolipoprotein (26–28) and enhancement of its degradation by unsaturated fatty acid (29) or overloaded cholesterol (30). Previous studies have suggested that ABCA1 phosphorylation is involved in its stabilization (31, 32). A PDZ adaptor protein α1-syntrophin was shown to stabilize ABCA1 (33).

In the present study, we attempted to examine the underlying mechanism for inhibition of ABCA1 activity by probucol. Probucol did not influence the transcription and intracellular

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[1] The abbreviations used are: HDL, high density lipoprotein; ABCA1, ATP-binding cassette transporter A1; LDL, low density lipoprotein; PBS, phosphate-buffered saline; apoA-I, apolipoprotein A-I; BSA, bovine serum albumin; CE, cholesteryl ester; MEM, modified Eagle's essential medium; PMSF, phenylmethylsulfonyl fluoride.

distribution of ABCA1 but suppressed its degradation by calpain to increase inactive ABCA1 in the plasma membrane.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Probucol (4,4′-(isopropylidenedithio)bis [2,6-di-*tert*-butylphenol]) was a generous gift from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). Calpain and calpeptin were from Calbiochem. [1,2-^{14}C]Cholesteryl oleate and [^{3}H]acetic acid were obtained from Amersham Biosciences, and iodine-125 (^{125}I) was from PerkinElmer Life Sciences.

Lipoprotein and Apolipoprotein—Lipoproteins were isolated from fresh human plasma by sequential ultracentrifugation in sodium bromide at a density of 1.006–1.063 g/ml for LDL and 1.125–1.21 g/ml for HDL. Lipoprotein-free plasma protein fraction was collected as a bottom fraction with a density of 1.21 g/ml. All plasma fractions were thoroughly dialyzed against 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl (PBS). Apolipoprotein A-I (apoA-I) was isolated from the human HDL fraction by delipidation followed by anion exchange column chromatography in 6 M urea as described previously (34). ApoA-I was dissolved in PBS before use in experiments according to the methods described previously (34).

Preparation of Probucol-containing LDL and Probucol-carrying Bovine Serum Albumin (BSA)—To deliver probucol to the cells, probucol-containing LDL was prepared according to the method described previously (23, 35). Lipid microemulsion was prepared by sonicating egg phosphatidylcholine (Avanti) and triolein (Wako Pure Chemicals) with and without probucol. LDL (15 mg of protein) was incubated at 37 °C for 48 h with the microemulsion containing or not containing probucol in the presence of lipid-free plasma fraction (1.5 g of protein), dithionitrobenzoic acid (2 mM), aprotinin (20 units/ml), gentamycin (0.1 mg/ml), EDTA (0.5 mM), and NaN_3 (0.1% w/v). The mixture was applied to a dextran sulfate-cellulose column to recover LDL as the bound fraction eluted with 0.5 M NaCl. LDL was further purified by ultracentrifugal floatation at a density of 1.063 g/ml and thoroughly dialyzed against PBS. To label cholesteryl ester (CE) in LDL, the emulsion was prepared with [^{14}C]cholesteryl oleate (30 μCi), and the same procedure was applied to prepare the labeled LDL containing probucol. Alternatively, probucol was conjugated with BSA (fatty acid-free, Sigma). 250 μg of probucol was solubilized in 125 μl of methanol and incubated with 10 ml of 10% BSA (w/v) for 1 h at 37 °C.

Cell Culture and Loading with LDL—WI-38 human fibroblast cells (RIKEN Cell Bank) (36) were grown at 37 °C in Eagle's minimum essential medium (Sigma) with 10% fetal calf serum (HyClone Laboratories, Inc.), 5 units/ml penicillin, and 5 μg/ml streptomycin (Invitrogen). Cells were seeded into a 35-, 60-, or 100-mm dish at a density of 1.5×10^5 cells/ml. When the cells were grown to 80% of a confluent stage, the probucol-containing LDL (0–50 μg as LDL protein/ml) or probucol-carrying BSA (0.2%, w/v) was added for 24-h incubation to load probucol in the cells.

Cellular Lipid Release Assay—The probucol-loaded cells prepared as described above were washed and maintained in the lipoprotein-free medium for the next 24 h prior to any further experiments. The cells were incubated for 24 h with various amounts of apoA-I in the medium containing 0.02% BSA. Lipid was extracted from the medium with chloroform:methanol (2:1, v/v) and cells with *n*-hexane:2-propanol (3:2, v/v), and total cholesterol, free cholesterol, and choline-phospholipid were determined by colorimetric enzymatic assay system (Kyowa Medics) (13).

Determination of Probucol in the Cells—Probucol in the cell lipid extracts was measured by reverse-phase high performance liquid chromatography according to the method described by Satonin and Coutant (37). The cell extracts from the medium of a 60-mm dish were dissolved in acetonitrile, hexane, 0.1 M ammonium acetate (90:6.5:3.5, v/v/v) and injected into a Deltapak C_{18} reversed-phase column (150 × 3.9 mm, 300 A; Waters). The mobile phase was acetonitrile:water (85:15, v/v) with the flow rate at 1.5 ml/min, and the probucol was detected by absorbance at 240 nm.

Evaluation of the Synthesis of Lipids—WI-38 cells at 80% of confluent stage in 35-mm dishes were washed with PBS and cultured in 1 ml of modified Eagle's essential medium (MEM) containing 0.2% BSA with or without probucol. After replacement with 1 ml of fresh medium, the cellular lipid was labeled by incubating for 2 h with 20 μCi/ml [^{3}H]acetic acid. Lipid was extracted with *n*-hexane:2-propanol (3:2, v/v), and radioactivity was determined in cholesterol, phosphatidylcholine, sphingomyelin, and cholesteryl ester after separation by thin layer chromatography.

Determination of Cholesterol Distribution to the Plasma Membrane—To determine traffic of newly synthesized cellular cholesterol moved to the cell surface, the cells were treated with 0.2% BSA carrying and not carrying probucol for 24 h and were labeled with [^{3}H]acetic acid for 2 h. To determine distribution of LDL-derived cholesterol to the cell surface, the cells were incubated for 24 h with [^{14}C]CE-LDL (50 μg of protein/ml) containing or not containing probucol, washed with PBS, and incubated with MEM containing heparin (1400 IU/ml) for 45 min at 4 °C to remove surface-bound LDL. The labeled cells were quickly washed with PBS, fixed with 1% glutaldehyde at room temperature for 10 min, and washed with PBS to remove the fixative reagent. The cells were then incubated with cholesterol oxidase (1 unit/ml) in MEM for 1 h to allow the conversion of cholesterol to cholestenone in the plasma membrane (38). After the cells were washed with PBS, cellular lipids were extracted, and radioactivity was determined for cholesterol, cholestenone, and cholesteryl ester separated by thin layer chromatography.

Subcellular Membrane Fractionation—Bulk membrane fraction was prepared as follows. Cells in 100-mm dishes were harvested and treated with 5 mM Tris-HCl (pH 7.5) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM benzamidine) for 30 min on ice with vortexing every 10 min. The cell debris and nuclei were removed by centrifugation at 650 × *g* for 10 min at 4 °C, and the supernatant was centrifuged at 444,000 × *g* for 40 min at 4 °C. Subcellular membrane subfractions were prepared as follows. The cell pellet after centrifugation at 600 × *g* for 10 min was lysed with cold extract solution (0.02 M boric acid, 0.3 mM EDTA, 1 mM PMSF, 1 mM benzamidine, and protease inhibitor cocktails (Sigma), pH 10) for 15 min on ice with vortexing every 5 min. The cell debris and nuclei were discarded by centrifugation at 650 × *g* for 10 min at 4 °C, and the supernatant was centrifuged at 12,000 × *g* for 1 h at 4 °C. The pellet was harvested, and the supernatant was further centrifuged at 290,000 × *g* for 30 min at 4 °C. The second pellet was harvested, and the supernatant was treated with 10% trichloroacetic acid to precipitate the protein.

Western Blotting—The membrane fraction and cellular subfractions were resuspended in 50 mM Tris-HCl (pH 7.5) containing 5 mM EDTA, 10 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 1% Triton X-100, and 1% protease inhibitor cocktails (Sigma) and were sonicated for 5 s. After determination of the protein content by a BCA method (Pierce), the fractions were dissolved in 9 M urea, 2% Triton X-100, 1% dithiothreitol and were developed in 6 or 15% (w/v) polyacrylamide gel electrophoresis in the presence of 10% SDS, respectively, and the proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) by semidry blotter in blotting buffer (25 mM Tris-HCl, 0.2 M glycine, and 10% methanol (v/v)) for 3.5 h. The membrane was blocked with 5% skim milk in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20 and was probed with the rabbit antiserum against the C-terminal peptide of human ABCA1 (26, 39), rabbit anticaveolin-1 (N-20) (Santa Cruz Biotechnology), anti-GLUT-1, mouse anti-integrin β_1 (CHEMICON International, Inc.), anti-GM130, and anti-Bip/GRP78 (BD Transduction Laboratories, BD Biosciences), respectively. The immunoreactive proteins were visualized by ECL or the ECL Plus system (Amersham Biosciences).

ABCA1 Degradation Rate—WI-38 cells in 100-mm dishes were incubated in 6 ml of the medium with LDL (300 μg of protein) containing or not containing probucol as described above. Cells were washed three times with PBS and incubated at 37 °C in MEM containing 20 ng/ml cycloheximide (Wako Pure Chemicals). ABCA1 in the cell membrane fraction was detected by immunoblot analysis as described above. For studying the calpain-mediated proteolysis of ABCA1, the experiment was performed according to the method described by Wang *et al.* (27). Cells were washed three times with PBS and placed on ice for 10 min. Then cells were permeabilized by incubating on ice for 15 min with 80 μg/ml digitonin in MEM. The cells were washed twice with PBS and incubated for 20 min at room temperature with μ-calpain (0.1 μM) in MEM containing 2 mM $CaCl_2$. The cells were lysed with 3 ml of buffer (5 mM Tris-HCl, pH 7.5, 1 mM PMSF, and 1 mM benzamidine) containing 40 μg/ml calpeptin. The ABCA1 in the cell membrane fraction was analyzed by using immunoblot analysis.

RNA Extraction and Real Time Quantitative PCR—Total RNA was extracted from cells by using RNA extraction reagent (Isogen, Nippon Gene). After contaminated genomic DNA was digested with DNase I (Takara Shuzo Co.), first standard cDNA was synthesized by a SuperScript™ preamplification system (Invitrogen) from 2 μg of the total RNA. PCR was performed by using primers (sense and antisense) for cDNA 5′-GAA CTG GCT GTG TTC CAT GAT-3′ and 5′-GAT GAG CCA GAC TTC TGT TGC-3′ (for ABCA1) and 5′-ATG GTG GGA ATG GGT CAG AAG-3′ and 5′-CAC GCA GCT CAT TGT AGA AGG-3′ (for β-actin)

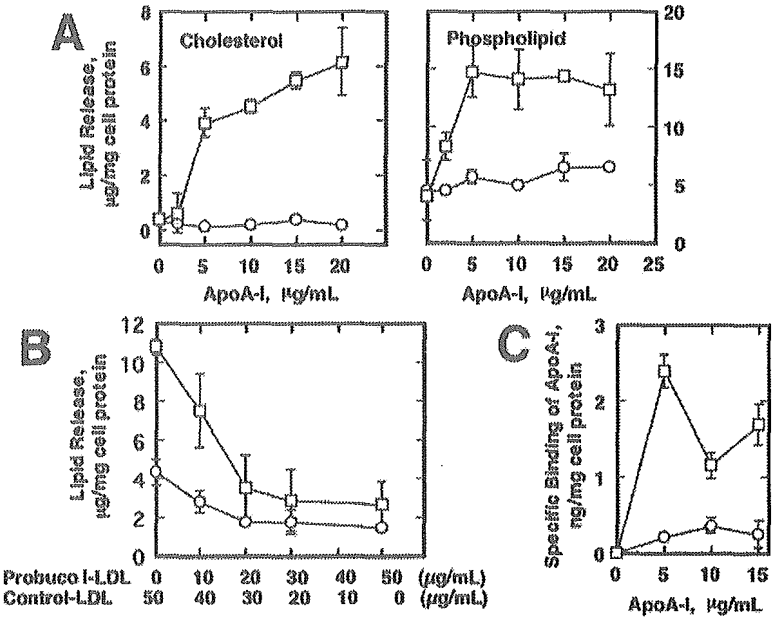

FIG. 1. **Effect of probucol on the apoA-I-mediated cellular lipid release.** WI-38 cells were incubated with LDL containing probucol or not containing probucol (50 μg of LDL protein/35 mm-dish) at 37 °C for 24 h and equilibrated for distribution of cholesterol and probucol by further incubation in 0.2% BSA for 24 h. Lipid-free apoA-I was added to the cells and incubated for 24 h. *A*, the *left-hand panel* shows release of cholesterol into the medium from the control cells (*squares*) and the probucol-treated cells (*circles*). The *right-hand panel* shows the release of choline-phospholipid from the control and probucol-treated cells (*squares* and *circles*, respectively). *B*, dose-dependent effect of probucol. The dose of probucol-containing LDL was increased (0, 10, 20, 30, 40, and 50 μg of LDL protein/35-mm dish) as the control LDL was decreased to maintain the total LDL protein at 50 μg. The release of cholesterol (*circles*) and choline-phospholipid (*squares*) was measured as described above. *C*, the ^{125}I-labeled apoA-I was incubated at 0 °C for 2 h with the WI-38 cells pretreated with LDL containing probucol or not as described above. Specific binding of ^{125}I-apoA-I was calculated by subtracting the binding after displacement with cold apoA-I from the total binding of the labeled apoA-I as described in the text for the control-LDL-treated (*squares*) and probucol-LDL-treated (*circles*) cells. The *data points* represent the average ± S.E. of an assay performed in triplicate.

(synthesized by Sawady Technology Co., Ltd.). The quantification of ABCA1 and β-actin mRNA was achieved using SYBR Green PCR master mix reagent in an ABI PRISM 7700 sequence detection system (Applied Biosystems Japan).

^{125}I-Apolipoprotein A-I Binding to the Cell Surface—Specific binding of ^{125}I-labeled apoA-I was estimated as displaceable binding by excessive cold apoA-I (23). ApoA-I was dissolved as 1 mg in 20 ml of the 0.1 M PBS (pH 6.5) and incubated at 37 °C for 1 h. ^{125}I (2 mCi) and 15 beads of IODO-BEAD® iodination reagent (Piece) were added to the solution, and the labeling reaction was carried out with stirring for 15 min at room temperature. The solution was concentrated by using the Ultrafree-15 centrifugal filter device (Millipore) with centrifugation at 700 × *g* for 1.5 h at 4 °C. The analysis of product by polyacrylamide gel electrophoresis showed that 94% of the total radioactivity was recovered in apoA-I and that the specific radioactivity was 2367, 608 cpm/μg of protein. After treatment with LDL containing or not containing probucol, cells in 35-mm dishes were incubated with various concentrations of ^{125}I-apoA-I at 0 °C for 2 h in 500 μl of MEM (pH 7.4, containing 25 mM HEPES, 0.2% BSA). The cells were chased twice by the medium with or without 50 μg/ml non-labeled apoA-I at 0 °C for 4 h and washed with cold PBS. The cell-bound radioactivity was recovered in 1 ml of 0.5 M NaOH.

RESULTS

Consistent with our previous reports (23, 24), probucol inhibited the apoA-I-mediated cellular lipid release (Fig. 1*A*). This effect was dose-dependent with respect to the dose of probucol-containing LDL (Fig. 1*B*). Probucol also inhibited displaceable binding of ^{125}I-apoA-I (Fig. 1*C*). Dose-dependent increase of cellular probucol content was observed within the range of the dosage of the probucol-containing LDL employed in the present experiment (Fig. 2*A*). Loading of cholesterol to the cells via LDL was not influenced by the presence of probucol in LDL (Fig. 2*B*).

The effect of probucol on lipid biosynthesis was examined. To avoid the effect of lipid loading via LDL, probucol was given to the cells as a BSA-probucol conjugate. Probucol also inhibited the apoA-I-mediated lipid release even when it was given directly by this procedure (Fig. 3*A*). In this condition, incorporation of acetate was somewhat increased by probucol in all of the lipid fractions tested, including free cholesterol, cholesteryl ester, phosphatidylcholine, and sphingomyelin (Fig. 3*B*).

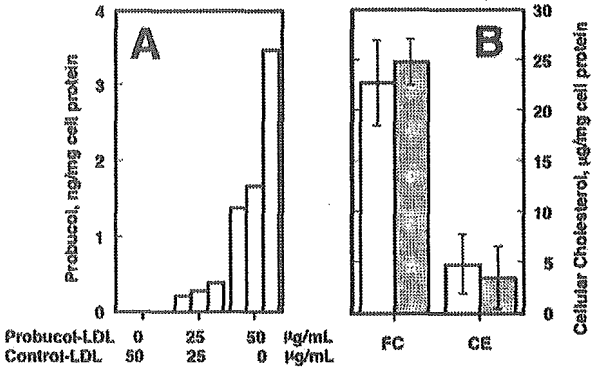

FIG. 2. **Accumulation of LDL-derived cholesterol and probucol in WI-38 cells.** *A*, WI-38 cells were incubated with probucol-free or probucol-containing LDL (total of 50 μg of protein) in 3 ml of medium within a 60-mm dish for 24 h. Probucol accumulated in the cells was measured using reverse-phase high pressure liquid chromatography as described under "Experimental Procedures." Each *bar* represents an individual experimental data point. *B*, the accumulation of cholesterol in WI-38 by incubating with probucol-free LDL (*open bar*) and probucol-carrying LDL (*dot-filled bar*) was measured. LDL (50 μg of LDL protein/35-mm dish) was incubated with cells at 37 °C for 24 h. The cellular cholesterol was determined as described under "Experimental Procedures." The values are the average and S.E. of triplicate assays. *FC*, free cholesterol.

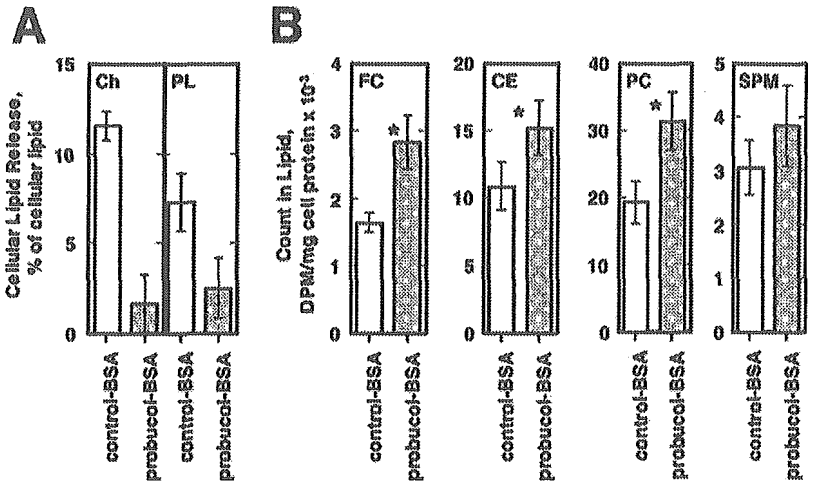

FIG. 3. **Effect of probucol on cellular lipid synthesis.** *A*, probucol-carrying or probucol-free 10% BSA was added to the medium to make the final BSA concentration 0.2% and incubated for 24 h at 37 °C. ApoA-I was added to the cells and incubated for another 24 h. The cholesterol (*Ch*) and phospholipid (*PL*) in the medium were measured as described under "Experimental Procedures." *B*, WI-38 cells were loaded with probucol as described above, followed by labeling with [^{3}H]acetic acid (20 μCi/ml) for 2 h. The cellular lipids were extracted and separated by thin layer chromatography, and radioactivity was determined in the fractions of free cholesterol (*FC*), cholesteryl ester (*CE*), phosphatidylcholine (*PC*), and sphingomyelin (*SPM*). Each *bar* represents the average and S.E. of three data points. *Dot-filled columns* and *open columns* indicate the data for probucol-treated and probucol-free cells, respectively. *Asterisks* indicate a significant increase from the control ($p < 0.05$).

Cellular cholesterol is distributed throughout 60–80% of the plasma membrane (40), and cholesterol accounts for as much as 30–40% of lipid molecules in the plasma membrane (41). Transport of cholesterol to the plasma membrane is differentially regulated for the newly synthesized and the LDL-derived cholesterol molecules. Therefore, distribution of cholesterol from these different sources was examined with respect to the effect of probucol. Cellular cholesterol was labeled by incubating with [^{3}H]acetic acid or by incorporating [^{14}C]CE-LDL, and cell surface cholesterol was probed by extracellular cholesterol oxidase. As shown in Fig. 4, probucol caused a small decrease in relative distribution of *de novo* synthesized cholesterol to the surface, probably because of the apparent increase of the synthesis shown in Fig. 3*B*, but did not cause any significant difference in the LDL-derived cholesterol. Based on the results above, it is unlikely that the inhibitory effect of probucol on the HDL assembly reaction is related to alteration of cellular cholesterol metabolism.

We analyzed the expression of ABCA1. The message of ABCA1 was not influenced by probucol at all (Fig. 5*A*). However, probucol increased ABCA1 as analyzed by immunoblotting of the membrane fraction, whereas there was no change in integrin β_1 and GLUT-1 (Fig. 5*B*). The initial immunocytochemical studies suggested that endogenously expressed human ABCA1 was localized in the plasma membrane (7, 42, 43), so the effect of probucol on intracellular distribution of ABCA1 was investigated. The cell membrane was fractionated as described under "Experimental Procedures," and each fraction was analyzed by immunoblotting for ABCA1 and membrane marker proteins. As demonstrated in Fig. 5*C*, ABCA1 was detected along with a plasma membrane marker, integrin β_1, predominantly in the pellet fraction that was centrifuged at 12,000 × *g* for 60 min (*lanes 1* and *5*). Caveolin-1 was also recovered mainly in this fraction. In contrast, a significant amount of a marker for endoplasmic reticulum, Bip/GRP78, was found in the supernatant fraction centrifuged at 290,000 × *g* for 30 min (Fig. 5*C*, *lanes 3* and *4* and *lanes 7* and *8*). A Golgi marker, GM130, was also distributed to this fraction. Probucol did not influence this pattern of distribution of ABCA1 and marker proteins. Thus, ABCA1 is predominantly present in the plasma membrane, and probucol does not significantly alter

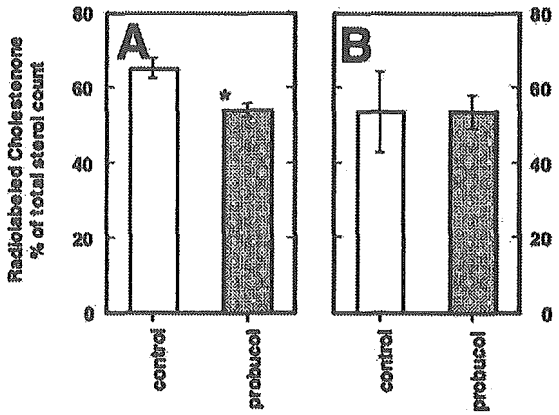

FIG. 4. **Determination of cholesterol content in plasma membrane by cholesterol oxidase.** WI-38 cells were incubated with 0.2% probucol-free or probucol-carrying BSA for 24 h and then were radiolabeled with [^{3}H]acetic acid (20 μCi/ml) for 2 h (*A*) or were incubated with LDL (50 μg protein/ml) containing [^{14}C]cholesteryl ester and probucol for 24 h at 37 °C (*B*). The cholesterol on the cell surface was probed by extracellular cholesterol oxidase as described under "Experimental Procedures." The results are displayed as the percentage of radioactive cholestenone within total cellular sterol. Results represent the mean ± S.D. of triplicate determinations of a representative experiment. An *asterisk* indicates a significant difference from the control ($p < 0.05$).

the traffic of ABCA1. ABCA1 and caveolin-1 may also be somewhat recovered with the Golgi-endoplasmic reticulum fractions, which is consistent with previous findings (44, 45).

To study the underlying mechanism for the increase of ABCA1 without changing its message, degradation of ABCA1 in the cell was examined. ABCA1 was analyzed by immunoblotting in the presence of cycloheximide. A decrease in ABCA1 was apparent at 30 min, and ABCA1 decayed throughout the incubation up to 120 min (Figs. 6*A* and 7*A*). Probucol apparently slowed the rate of the decay of ABCA1 (Figs. 6*A* and 7*A*).

To examine the effect of probucol on proteolytic degradation of ABCA1, the susceptibility of ABCA1 to protease was observed after the cells were permeabilized by digitonin. To exclude a possibility that probucol may inhibit the membrane

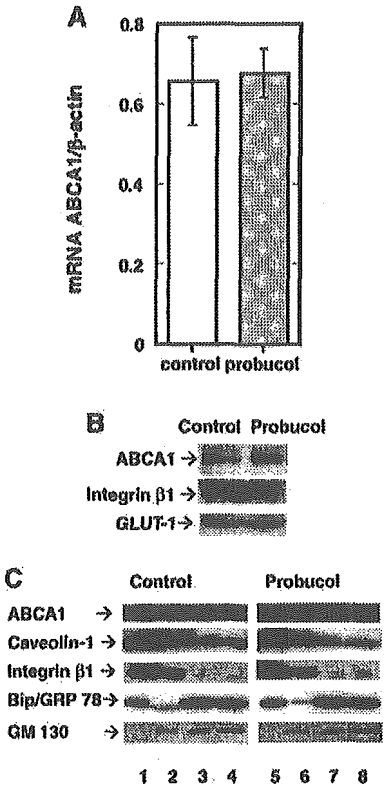

FIG. 5. **Effect of probucol on ABCA1.** WI-38 cells were incubated with LDL containing probucol or not for 24 h. *A*, specific messages of ABCA1 and β-actin were quantified by real time PCR. The ABCA1 message is standardized for the β-actin mRNA in the figure. The data represent the average ± S.E. of an assay performed in triplicate. *B*, total membrane fraction was prepared from the probucol-treated and control cells as described in the text. Fifty μg of the membrane protein was analyzed by immunoblotting for ABCA1, GLUT-1, and integrin β_1. *C*, the subcellular membrane fractions were prepared as described under "Experimental Procedures" as the pellet of the first centrifugation (12,000 × *g* for 60 min), the pellet of the second centrifugation (290,000 × *g* for 30 min), the supernatant of the first centrifugation, and the supernatant of the second centrifugation. Each fraction was analyzed by immunoblotting for ABCA1, caveolin-1, integrin β_1 (plasma membrane marker), Bip/GRP78 (endoplasmic reticulum marker), and GM130 (Golgi marker). *Lanes 1* and *5*, 55 μg of protein of the pellet fraction of the first centrifugation. *Lanes 2* and *6*, 55 μg of protein of the pellet fraction of the second centrifugation. *Lanes 3* and *4* and *lanes 7* and *8*, 55 μg of protein precipitated with 10% trichloroacetic acid from the supernatant of the second centrifugation.

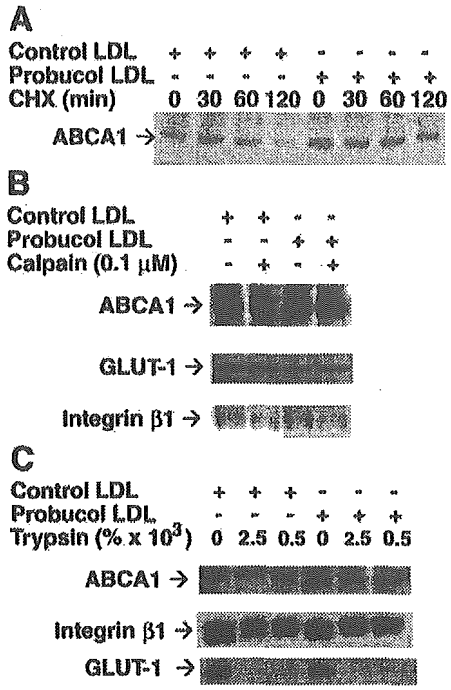

FIG. 6. **Effect of probucol on ABCA1 degradation.** *A*, WI-38 cells were pretreated with control or probucol-containing LDL. The cells were then incubated in the presence of cycloheximide (*CHX*) (20 ng/ml) for the indicated times, and membrane ABCA1 was analyzed by immunoblotting. 55 μg of the membrane protein was applied to each *lane*. *B* and *C*, WI-38 cells pretreated with control or probucol-containing LDL were permeabilized by incubating with 80 μg/ml digitonin on ice for 15 min. The cells were then incubated with μ-calpain (0.1 μM) in MEM containing 2 mM $CaCl_2$ for 20 min at room temperature (*B*) or with trypsin at the indicated concentration for 3 min at room temperature (*C*). Membrane ABCA1, GLUT-1, and integrin β_1 were analyzed by immunoblotting assay.

permeabilization by digitonin, cells were examined with immunostaining for β-tubulin after the permeabilization treatment. There was no difference between the cells treated with LDL containing probucol and no probucol (data not shown). ABCA1 in the cells treated with probucol became resistant to μ-calpain (a ubiquitously expressed subtype of calpain) (Figs. 6*B* and 7*B*). In contrast, probucol did not influence degradation of ABCA1 by trypsin (Figs. 6*C* and 7*C*). GLUT-1 was insensitive to calpain, whereas integrin β_1 was susceptible (Figs. 6*B* and 7*B*). Probucol did not influence digestion of integrin β_1 and GLUT-1, either by calpain or by trypsin (Fig. 6, *B* and *C*, and Fig. 7, *B* and *C*). The data provided direct evidence that physiological degradation of ABCA1 by calpain is inhibited by probucol.

DISCUSSION

The underlying mechanism for the inhibitory effect of probucol on the function of ABCA1 was investigated. The results of the study are summarized as follows. 1) Probucol inhibited the events mediated by ABCA1 such as apolipoprotein-mediated cellular lipid release and apolipoprotein binding to the cells. 2) Probucol did not interfere with transcription and intracellular trafficking of ABCA1, and ABCA1 was predominantly found in the plasma membrane even in the probucol-treated cells as judged by biochemical parameters. 3) Probucol made ABCA1 resistant to calpain-mediated degradation and consequently increased its cellular level. 4) Probucol did not influence cellular lipid accumulation via LDL and cholesterol distribution to the cell surface but rather enhanced lipid biosynthesis. We thereby concluded that probucol inactivated ABCA1 in the plasma membrane with respect to its functions and its susceptibility to proteolysis.

Probucol was used as a lipid-lowering drug for years before statins became available. This drug was known for its apparent clinical effect of regression of cutaneous and tendinous xanthomas more than expected from the reduction of plasma LDL (46). Because probucol is characterized for its strong antioxidative nature, it was expected to work as an antioxidant against oxidative modification of plasma lipoprotein to prevent development of atherosclerotic vascular lesions and even to cure them. This hypothesis was indeed strongly supported by several experimental approaches using animal models for atherosclerosis (20, 21). However, this drug has also been known for its strong effect of lowering plasma HDL. What is more puzzling is that reduction of HDL by probucol is sometimes seriously aggravated by fibrates, which are otherwise expected to raise HDL in plasma (22). Thus, probucol has been a very

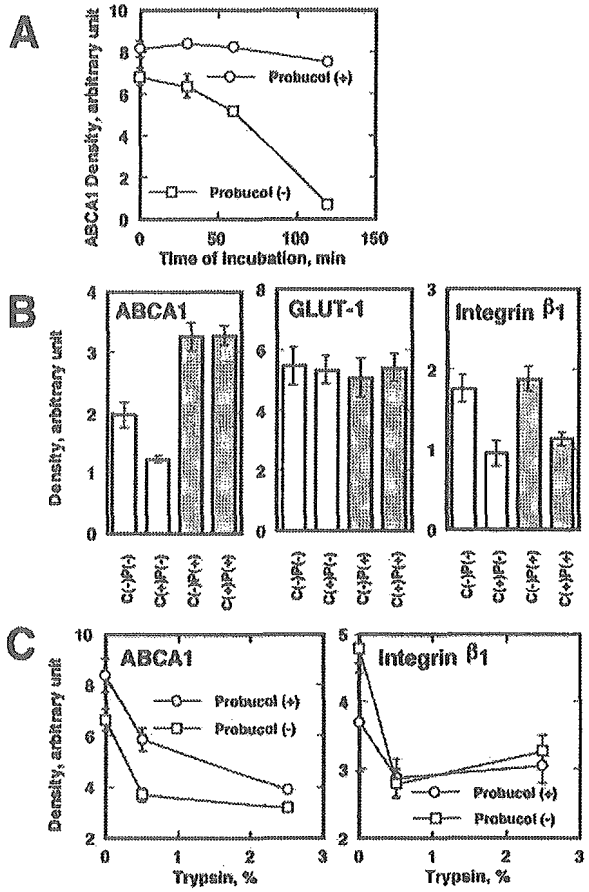

FIG. 7. **The results of density scanning of the Western blotting bands in the experiments represented by Fig. 6.** *Panels A, B,* and *C* correspond with *A, B,* and *C* of Fig. 6, respectively. In *B, C* and *P* indicate calpain and probucol, respectively. The density of each band was quantitated by digital scanning in an Epson GT9500. Data points represent mean ± S.E. of three experiments.

controversial drug, and it lost its popularity in the market as statins took over.

We discovered that probucol inhibits the apolipoprotein-mediated cellular lipid removal and generation of HDL as well as the binding of apolipoprotein to the cells (23). This finding is very similar to the observation with the cells of patients with Tangier disease, a familial HDL deficiency (3, 4). We thereby speculated that this is a reason for probucol to reduce HDL, and accordingly the apolipoprotein-mediated generation of HDL with cellular lipid is a main source of plasma HDL. We further investigated an *in vivo* effect of probucol by using mouse models. Probucol inhibited the production of HDL in mice, and the kinetic analysis of plasma HDL revealed the enhanced clearance of HDL apoprotein by probucol but no difference in the HDL-lipid clearance (24), an exact analogy to the behavior of HDL in Tangier disease patients (47), indicating that probucol inhibits the reaction to generate HDL that is lacking in patients with Tangier disease. The use of probucol in the lecithin:cholesterol acyltransferase-deficient mice showed that cholesterol accumulated only in the liver, suggesting that the liver is the major organ where generation of HDL by this mechanism takes place (48). ABCA1 was identified as a protein essentially responsible for this reaction, and the action of probucol on ABCA1 has become a subject of study.

In the current study, we focused on the effects of probucol on ABCA1 with respect to its transcription and trafficking as well as its role in mediating apoA-I binding and cellular lipid release by apolipoprotein. We also investigated its effect on proteolytic degradation of ABCA1, another main mechanism for regulating the level of ABCA1 in cells. Interestingly, there was no effect of probucol either on transcription or on trafficking of ABCA1 as judged by biochemical analysis. The cellular ABCA1 was rather significantly increased, and this was apparently because of the decrease of susceptibility of ABCA1 to proteolysis by calpain, a physiological regulation pathway for ABCA1. Thus, probucol inactivated not only functional aspects of ABCA1 but also its clearance system. Probucol is a very hydrophobic compound, and accordingly it is almost water-insoluble. Therefore, it is almost exclusively carried by lipoprotein in plasma and distributes in the membrane when cleaved to cells (37). Because almost all ABCA1 is recovered in plasma membrane, probucol is likely to act on ABCA1 in the plasma membrane. The decrease of protease susceptibility is not observed for trypsin, so the effects of probucol seem to induce a certain specific conformational alteration of ABCA1 to inactivate this protein against calpain, presumably at the membrane-spanning regions.

Probucol did not significantly influence intracellular lipid metabolism except that incorporation of [^{3}H]acetic acid into various lipid fractions increased somewhat, which may require further investigation. Thus, the effect of probucol on ABCA1 is unlikely to be a secondary phenomenon to its effect on cellular lipid metabolism.

There are no previous reports of any chemical compound acting in such a manner to inactivate membrane proteins. Further studies are required to elucidate the detail of the mechanism by which probucol inactivates ABCA1 in the membrane. This would provide important information about the reaction mechanism of ABCA1, cellular cholesterol homeostasis, and generation of HDL.

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Promoter polymorphism in fibroblast growth factor 1 gene increases risk of definite Alzheimer's disease

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Abstract

Fibroblast growth factor 1 (FGF1, also known as acidic FGF) protects selective neuronal populations against neurotoxic effects such as those in Alzheimer's disease (AD) and HIV encephalitis. The FGF1 gene is therefore a strong candidate gene for AD. Using the promoter polymorphism of the FGF1 gene, we examined the relationship between AD and the FGF1 and apolipoprotein E (APOE) genes in 100 Japanese autopsy-confirmed late-onset AD patients and 106 age-matched non-demented controls. The promoter polymorphism (−1385 A/G) was significantly associated with AD risk. The odds ratio for AD associated with the GG vs non-GG genotype was 2.02 (95% CI = 1.16–3.52), while that of ε4 vs non-ε4 in APOE4 gene was 5.19 (95% CI = 2.68–10.1). The odds ratio for APOEε4 and FGF1 GG carriers was 20.5 (95% CI = 6.88–60.9). The results showed that the FGF1 gene is associated with autopsy-confirmed AD.

Keywords: Definite Alzheimer's disease; Fibroblast growth factor 1 gene; Promoter polymorphism; Association study; APOE; Risk factor

Alzheimer's disease (AD; MIM#104300) is the most common cause of dementia in mid- to late-life. Studying the factors that influence the risk of developing AD may lead to the identification of those at high risk for developing it, strategies for prevention or intervention, and clues to the cause of the disease. Both genetic and environmental factors have been implicated in the development of AD [1], but the cause of AD remains unknown, and no cure or universally effective treatment has yet been developed [2]. Even the diagnosis is difficult. A definitive diagnosis depends on analysis of neuritic plaques and neurofibrillary tangles found in brain tissue [3]. Given the recognition that AD constitutes a heterogeneous disorder, identification of established risk factors would be difficult using conventional methods.

Fibroblast growth factor 1 (FGF1, also known as acidic FGF) is a member of the fibroblast growth factor family that possesses broad mitogenic and cell survival activities and is involved in a variety of biological processes [4]. FGF1 protects selective neuronal populations against neurotoxic effects such as those in Alzheimer's disease [5,6] and HIV encephalitis [7]. Immunohistochemical examination of postmortem brain tissue of AD revealed that FGF1 was specifically expressed in a subpopulation of reactive astrocytes surrounding senile

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plaques. Such upregulation of FGF1 expression might be related to the presence of reactive astrocytes rather than β-amyloid protein deposition [8,9]. Recent studies suggest that FGF1 upregulates APOE synthesis and subsequently HDL production in reactive astrocytes in an autocrine or paracrine manner, and exerts its effect after central nervous system (CNS) damage through APOE secretion [10,11]. Besides, the fact that FGF1 expression is lower in the hippocampal formation than in motoneurons suggests that FGF1 contributes to the selective vulnerability of neurons in the entorhinal cortex in AD, and altered patterns of FGF1 immunoreactivity may play an important role in the pathophysiological processes of AD [6,12]. The FGF1 gene is therefore a strong candidate gene for AD. However, there are no reports regarding the association of FGF1 gene polymorphism with AD. Therefore, we investigated whether FGF1 gene polymorphism could contribute to risk in a limited subgroup of AD (autopsy-confirmed AD).

Subjects and methods

The Ethics Committee of Ehime University School of Medicine approved the study protocol. Patients were selected based on the NINCDS-ADRDA criteria for definite AD, and non-demented controls were rigorously evaluated for cognitive impairment using the Mini-Mental State Examination [3,13]. Brain and blood samples were obtained with informed consent from subjects in the Chubu and Kansai areas of Japan. A total of 100 unrelated late-onset AD (LOAD) patients had been diagnosed previously, and 106 controls (outpatients or healthy volunteers) were selected and matched for age and place of residence of the patients as described elsewhere [14,15]. The mean age ± SD (years) at the time of this study was as follows: 85.3 ± 6.0 for LOAD, 83.0 ± 4.9 for controls. Genomic DNA was extracted from the brain or peripheral blood using the phenol–chloroform method [16].

During screening for FGF1 gene mutation and polymorphism, we detected a common single nucleotide polymorphism (SNP) of −1385 G/A (C/T) (rs34011) in the promoter region. This polymorphism could easily be detected by PCR-RFLP using the restriction enzyme *Hha*I, where G and A, with respective frequencies of 0.65 and 0.35, were observed in our Japanese control population. The polymorphic region was amplified by PCR with the primers FGF1-F (5′-TCAAGC AATTCTCCTGCCTT-3′) and FGF1-R (5′-CCACTTCAAGGGATT ATGGTG-3′). PCR was carried out in a 25-μl reaction volume containing standard reaction buffer (1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris–HCl, pH 8.3), 200 μM each dNTP, 5 μM each primer, 0.5 U *Taq* DNA polymerase and 50 ng genomic DNA as a template with 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. PCR product size was 355 bp, and the G allele was digested by *Hha*I to 53 + 141 + 161 bp, and the A allele to 53 + 302 bp. DNA was electrophoresed on 2% agarose gels and visualized with ethidium bromide staining under UV light (Fig. 1). To investigate the contribution of the gene to sporadic LOAD, we compared allele frequencies between LOAD and normal control subjects. Because APOEε4 is a risk factor for AD, we stratified the population by ε4 carrier status. APOE genotyping was performed as described previously. Allelic and genotypic distribution were analyzed by the usual χ^2 test of association. The genotypic frequencies were compared by χ^2 test with the values predicted by the assumption of Hardy–Weinberg equilibrium in the sample. Values of $p < 0.05$ were considered significant. Odds ratios were calculated with two-tailed p values and 95% confidence intervals.

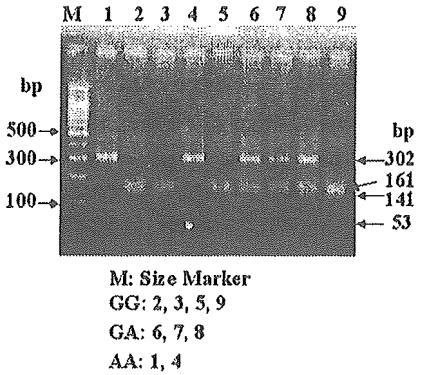

Fig. 1. Promoter polymorphism of FGF1. After amplification, PCR products were digested with *Hha*I and DNA was detected after electrophoresis on 2% agarose gels. Three genotypes of −1385 G/A (*Hha*I polymorphism) are shown: genotypes GG (lanes 2, 3, 5, and 9), GA (lanes 6–8), and AA (lanes 1 and 4).

Results

The PCR results were scored by two independent investigators who did not know whether each sample was from a case patient or a control. No intraobserver variability was found on repeated readings of the same gel, and the interobserver variability was less than 1%. All ambiguous samples were analyzed a second time.

The distribution of the three genotypes (GG, GA, and AA) reached Hardy–Weinberg equilibrium. The G allele was found in 75% of the 100 LOAD patients and 63% of the 106 control subjects. A significant association was observed between the −1385 G/A polymorphism and LOAD ($p < 0.03$; Table 1). We then examined the GG genotype as a risk factor for AD, considering the APOE status. As expected, APOEε4 conferred an increased risk for AD [odds ratio (OR) = 5.19]. OR in homozygotes for the G allele was 2.02 [95% confidence interval (CI) = 1.16–3.52]. However, the risk-increasing effect was smaller for −1385 G than for APOEε4 (Table 2). Four categories were defined by the presence (+) or absence (−) of a ε4 or GG genotype. The GG genotype alone showed an increased risk (95% CI: 1.81–7.69), and OR for APOEε4 and the GG genotype was 20.5 (95% CI: 6.88–60.9).

Discussion

To date, some polymorphisms of the FGF1 gene have been reported to associate with intracranial aneurysm [17]. However, functional role of the haplotype in its pathophysiology remains unclear. As the FGF1 gene contains alternative 5′-untranslated exons, the transcription is controlled by at least four distinct promoters in a tissue-specific manner [18–20]. Payson et al. [19] have reported that the sequence from −1614

Table 1
Genotype and allele numbers and frequencies for G/A polymorphism in promoter of FGF1

Group	Genotype (frequency)				Allele (frequency)	
n	AA	GA	GG	AA + GA	G	A
LOAD (100)	6 (0.06)	38 (0.38)	56 (0.56)*	44 (0.44)**	150 (0.75)	50 (0.25)***
Control (106)	14 (0.13)	51 (0.48)	41 (0.39)	65 (0.61)	133 (0.63)	79 (0.37)

LOAD, late-onset AD.
* $p < 0.03$.
** $p < 0.02$.
*** $p < 0.01$.

Table 2
Relative risk for interaction between APOEε4 and −1385 GG

		LOAD cases	Controls	Odds ratio	95% CI
	−1385 G/A				
	non-GG	44	65	Reference	
	GG	56	41	2.02	1.16–3.52
APOEε4					
−		52	90	Reference	
+		48	16	5.19	2.68–10.1
APOEε4	−1385 GG				
−	−	17	58	Reference	
−	+	35	32	3.73	1.81–7.69
+	−	18	11	5.58	2.21–14.1
+	+	30	5	20.5	6.88–60.9

APOEε4 (+), one or two copies of ε4: APOEε4 (−), no copies of ε4, 95% CI, confidence interval at 95% level.

to the FGF1 start site is sufficient to stimulate promoter activity. Therefore, it is reasonable to think that −1385 G/A polymorphism in the FGF1 promoter region can contribute the promoter activity. We performed an association study of the promoter polymorphism of the FGF1 gene.

We have evaluated definite LOAD as a relatively homogeneous case group. Our preliminary data suggest that the FGF1 gene, or a nearby gene, is an additional risk factor, independent of the APOE gene. Association studies often produce conflicting results. There are three possible reasons. First, this might be due to a type I statistical error, where there is a weak association between the polymorphism and the disease. Second, it might arise from the difference in genetic background between the American, French, Asian, and Japanese populations. In some studies, the AD group was made up of a mixture of familial and sporadic patients. We therefore tried to choose homogeneous subjects (autopsy-confirmed and late-onset AD) as much as possible. A third possibility could be linkage disequilibrium with other causative polymorphisms.

Patients with the GG genotype in this study had a higher risk of AD than those with the A allele. This indicates that the GG genotype in the promoter may influence the expression of FGF1 and could be involved in the selective vulnerability of neurons in AD. The results of this study support the hypothesis that FGF1 contributes to the selective vulnerability of neurons in the entorhinal cortex in AD, and altered patterns of FGF1 immunoreactivity may play an important role in the pathophysiological processes of AD [11,6,12]. This hypothesis should be further examined by functional analysis of FGF1 polymorphisms.

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Intracellular cholesterol mobilization involved in the ABCA1/apolipoprotein-mediated assembly of high density lipoprotein in fibroblasts

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Abstract **Differential regulation has been suggested for cellular cholesterol and phospholipid release mediated by apolipoprotein A-I (apoA-I)/ABCA1. We investigated various factors involved in cholesterol mobilization related to this pathway. ApoA-I induced a rapid decrease of the cellular cholesterol compartment that is in equilibrium with the ACAT-accessible pool in cells that generate cholesterol-rich HDL. Pharmacological and genetic inactivation of ACAT enhanced the apoA-I-mediated cholesterol release through upregulation of ABCA1 and through cholesterol enrichment in the HDL generated. Pharmacological activation of protein kinase C (PKC) also decreased the ACAT-accessible cholesterol pool, not only in the cells that produce cholesterol-rich HDL by apoA-I (i.e., human fibroblast WI-38 cells) but also in the cells that generate cholesterol-poor HDL (mouse fibroblast L929 cells). In L929 cells, the PKC activation caused an increase in apoA-I-mediated cholesterol release without detectable change in phospholipid release and in ABCA1 expression. These results indicate that apoA-I mobilizes intracellular cholesterol for the ABCA1-mediated release from the compartment that is under the control of ACAT. The cholesterol mobilization process is presumably related to PKC activation by apoA-I.**—Yamauchi, Y., C. C. Y. Chang, M. Hayashi, S. Abe-Dohmae, P. C. Reid, T-Y. Chang, and S. Yokoyama. **Intracellular cholesterol mobilization involved in the ABCA1/apolipoprotein-mediated assembly of high density lipoprotein in fibroblasts.** *J. Lipid Res.* **2004.** 45: **1943–1951.**

Supplementary key words ATP binding cassette transporter A1 • acylcoenzyme A:cholesterol acyltransferase • apolipoprotein A-I • protein kinase C

Cholesterol has various important biological functions, such as regulation of the structure and function of cellular membranes, covalent modification of protein, and biosynthesis of steroid hormones and bile acids as their precursors. Cellular cholesterol content and its distribution are therefore tightly regulated by various factors, and intracellular cholesterol trafficking is closely related to its cellular homeostasis. One of the sensing sites of cellular cholesterol level is the endoplasmic reticulum, where various important molecules for cholesterol homeostasis are located. Sterol regulatory element binding proteins (SREBPs) and their related elements are identified as a system to regulate various genes for cholesterol biosynthesis and its uptake. ACAT is also in the endoplasmic reticulum and functions to reduce excess free cholesterol by its esterification. On the other hand, cellular cholesterol is released to the extracellular environment primarily for its catabolism, because cholesterol is hardly metabolized in most somatic cells (1). This is also recognized as one of the crucial factors in cholesterol homeostasis in peripheral cells. ACAT reaction and cholesterol release are both active systems to protect cells from the membrane-toxic excess accumulation of free cholesterol.

Cellular cholesterol is removed in two distinct pathways by HDL to be transported to the liver for degradation to bile acids. Cellular cholesterol is actively released by lipid-free apolipoproteins that dissociate from HDL (2) to form new HDL particles with cellular phospholipid, whereas cholesterol molecules leave the cell surface to HDL by passive diffusion, which is enhanced by extracellular cholesterol esterification in HDL (1). It has been demonstrated that cells from patients with Tangier disease, a familial HDL deficiency, lack apolipoprotein-mediated lipid

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Abbreviations: apoA-I, apolipoprotein A-I; DOG, *sn*-1,2-dioctanoylglycerol; PKC, protein kinase C; PMA, phorbol 12-myristate-13-acetate; SREBP, sterol regulatory element binding protein.

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release (3), and mutations have been identified in the gene *abca1* of these patients (4–6). Numerous studies were carried out to characterize this gene and its product, ABCA1, including its overexpression by cDNA transfection (7, 8), cyclic AMP analog treatment (9, 10), and stimulation by liver X receptor and/or retinoid X receptor ligands (11, 12), and demonstrated that ABCA1 mediates the HDL assembly by apolipoprotein with cellular lipids. In addition, findings with knockout mice (13, 14) and transgenic mice (15) of *abca1* confirmed an essential role of this molecule in the generation of plasma HDL. It has thus been established that ABCA1 is a rate-limiting factor of apolipoprotein-mediated lipid release and subsequent HDL assembly. However, it remains to be addressed how this protein mediates the reaction.

We recently reported that apolipoprotein-mediated releases of cholesterol and phospholipid are differentially regulated. Fibroblast cell lines can apparently be categorized into three groups: cells that generate *1*) cholesterol-rich HDL, *2*) cholesterol-poor HDL, and *3*) no HDL, after apolipoprotein A-I (apoA-I) exposure (16). This report demonstrated that ABCA1 expression is required for apoA-I-mediated phospholipid release and for the subsequent generation of HDL particles, rather than a direct requirement for cholesterol release and increase of cholesterol content in the HDL. Caveolin-1 was previously shown to be involved in the enrichment of cholesterol in the HDL generated by the apolipoprotein-mediated reaction in certain types of cells (17–19). However, L929 cells, for example, abundantly express both ABCA1 and caveolin-1 and yet generate only cholesterol-poor HDL (16). Thus, regulation of cholesterol enrichment of the HDL generated by an ABCA1/apolipoprotein system seems multifactorial. An additional factor(s) may be required to induce cellular cholesterol release for the apolipoprotein/ABCA1 pathway. In this article, mobilization of intracellular cholesterol for its release by this pathway was investigated. We show that protein kinase C (PKC) and ACAT-1 activities are involved in regulating the rate of intracellular cholesterol mobilization for ABCA1-mediated cholesterol release by apoA-I.

MATERIALS AND METHODS

Materials

ApoA-I was prepared from fresh human plasma HDL as described (20). Phorbol 12-myristate-13-acetate (PMA) and 4α-PMA were purchased from Wako (Osaka, Japan), and *sn*-1,2-dioctanoylglycerol (DOG) was from Seikagaku Corporation (Tokyo, Japan). An ACAT inhibitor, F12511 (21), was a gift of Pierre Fabre Research (Castres Cedex, France) to T.Y.C.

Cell lines and cell culture

The fibroblast cell lines WI-38 (a human fibroblast cell line), L929 (a mouse fibroblast cell line), and COS-7 (a monkey fibroblast cell line) were incubated as described (16). Human embryonic kidney-derived cell line HEK293 and a clone of its stable human ABCA1-green fluorescent protein transfectant (293/2c) were maintained in DMEM with 10% FBS as reported (22, 23), and this clone has been extensively studied (22–24). ACAT-1-deficient CHO cells, AC29 (25), its parental 25RA cells (26), and AC29 stably expressing human ACAT-1 (AC29/hACAT1) were grown in a 1:1 mixture of DMEM and Ham's F12 supplemented with 10% FBS plus 10 μg/ml gentamycin. 25RA cells have a gain-of-function mutation in SREBP cleavage-activating protein, resulting in constitutive activation of the proteolytic cleavage of SREBPs (27). The AC29/hACAT1 cell line was generated by transfection of pcDNA3 (Invitrogen) harboring human ACAT-1 cDNA (1397–4011 bp region, including the full-length open reading frame) (28). The pCMV4 plasmid containing human ACAT cDNA K1 (28) was digested by *Sal*I and *Sma*I. The resulting human ACAT-1 cDNA fragment was subcloned into *Eco*RV sites of pcDNA3, and AC29 cells were transfected with the plasmid by using Lipofectamine reagent (Invitrogen). A stable clone was isolated by the selection of G-418 resistance and further verified by the presence of cytoplasmic cholesteryl ester lipid droplets as visualized with a phase-contrast microscope. The clone was designated AC29/hACAT1, and it showed expression of the 50 kDa human ACAT-1 as confirmed by Western blotting (data not shown). Its enzyme activity is described in **Table 1**.

Cellular lipid release

Cells grown at a confluent stage in six-well trays were incubated with or without apoA-I for the indicated periods of time in the presence of 0.1% fatty acid-free BSA, except that 0.02% BSA was used for HEK293 cells. After the incubation, lipid in medium and cells was extracted, and free cholesterol, total cholesterol, and choline-containing phospholipid were then determined enzymatically by the method described (10, 16). Alternatively, cellular lipids were radiolabeled with [^{3}H]cholesterol (NEN Life Science Products, Inc., Boston, MA) or with [^{3}H]choline chloride (NEN Life Science Products, Inc.) for 20–24 h, and the cells were incubated under the indicated conditions after washing with PBS. Cellular and medium lipids extracted were separated by TLC, and radioactivity of the desired lipid was determined by scintillation counting.

Measurement of the free cholesterol pool available for ACAT

The ACAT-accessible cholesterol pool in the cells was estimated by measuring the incorporation of [^{14}C]oleic acid into

TABLE 1. Cellular cholesterol and ACAT activity in CHO mutants examined

Variable	25RA	AC29	AC29/hACAT1
Lipid droplets	+	−	+
Total cholesterol	68.8 ± 2.9	35.9 ± 1.7	87.7 ± 4.3
Free cholesterol	43.8 ± 2.8	33.7 ± 1.2	62.7 ± 3.4
Cholesteryl ester	25.0 ± 1.9	2.2 ± 0.5	25.0 ± 1.4
Phospholipid	100.7 ± 5.7	116.2 ± 4.4	129.1 ± 6.2
Intact cell assay	10,388 ± 49	78 ± 4	11,488 ± 899
In vitro assay	40 ± 5	0 ± 0	89 ± 5

25RA, AC29, and AC29/hACAT1 cells were grown in medium containing 10% FBS. Cellular lipid contents were measured by enzymatic colorimetric assays as described in Materials and Methods after incubation of cells in medium with 0.1% BSA for 24 h. Cholesteryl ester was calculated by subtracting free cholesterol from total cholesterol. The data represent means ± SD of triplicate assays and are expressed as micrograms of lipid per milligram of cell protein. ACAT activity in these cells was determined by the intact cell ACAT assay and by the in vitro ACAT assay as described in Materials and Methods. These data represent the average ± variation between duplicate assays expressed as disintegrations per minute per milligram of cell protein for the intact cell assay or as picomoles per minute per milligram of cell protein for the in vitro assay.

cholesteryl ester in 1 h. After incubation of the cells in six-well trays at 37°C with or without stimulants (apoA-I, PMA, or DOG) for various periods of time in 0.1% or 0.02% (only for HEK293 cells) BSA-containing medium, the cells were further incubated in the presence of 1.5 or 1.0 μCi/ml [1-^{14}C]oleic acid (NEN Life Science Products, Inc.) for 1 h at 37°C in the same condition. After the cells were washed three times with ice-cold PBS, cellular lipids were extracted and separated by TLC to measure radioactivity in cholesteryl ester.

ACAT assays

ACAT activity was determined by two different methods: intact cell ACAT assay and in vitro ACAT assay. In the intact cell assay, cells grown in medium containing 10% FBS were incubated with [^{3}H]oleate in BSA for 20 min and the incorporation of [^{3}H]oleate into cholesteryl ester was measured as described (29). The in vitro ACAT assay was performed as described previously (30). Briefly, whole cell extract prepared by hypotonic shock was solubilized, and ACAT was then placed in mixed micelles. ACAT activity was probed by measuring the incorporation of [^{3}H]oleoyl-CoA into cholesteryl ester.

PKC assay

PKC activation was measured as described (24). Briefly, cells in a confluent stage in 100 mm dishes were incubated in the medium with 0.1% BSA for 20–24 h before stimulation by apoA-I (10 μg/ml) for various periods of time or with 160 nM PMA for 20 min as a positive control. The membrane fraction was prepared, and PKC activity in the membrane fraction (5 μg of protein) was determined by using a MESACUP Protein Kinase Assay Kit (Medical and Biological Laboratories) according to the manufacturer's instruction.

Immunoblotting of ABCA1

Total membrane fraction or total cell lysate was prepared, and ABCA1 was analyzed by immunoblotting with the rabbit antiserum against the C-terminal peptide of human ABCA1 as described (16, 31, 32). Consistency of protein loading was confirmed by Coomassie Brilliant Blue staining of the electrophoretic gels or by immunoblotting of β-actin using anti-β-actin monoclonal antibody (clone AC-74 from Sigma). The signal intensity of ABCA1 was measured with NIH Image 1.61 software, and fold change in ABCA1 level was analyzed.

RESULTS

Change of the cellular cholesterol pool available to ACAT as induced by apoA-I

To elucidate the mechanisms for the cholesterol enrichment of HDL generated by the apoA-I/ABCA1 pathway, we used WI-38 human fibroblasts, L929 mouse fibroblasts, and COS-7 monkey fibroblasts to represent the cells that generate cholesterol-rich HDL, cholesterol-poor HDL, and no HDL by apoA-I treatment, respectively. As we reported previously (16), WI-38 cells released both cholesterol and phospholipid, L929 cells predominantly released phospholipid, and COS-7 cells released neither cholesterol nor phospholipid upon incubation with apoA-I (**Fig. 1A, B**). The ratio of cholesterol to phospholipid in the conditioned medium was therefore higher in WI-38 cells than L929 cells (**Table 2**), reflecting the lipid profiles of the HDL fraction generated by these cells (16). The release of cholesterol and phospholipid by apoA-I from WI-38 was linear up to 24 h (Fig. 1C).

Change in the cellular ACAT-accessible cholesterol pool by apoA-I was estimated in these three fibroblast cell lines. The ACAT-accessible cholesterol pool was probed by measuring the incorporation of [^{14}C]oleic acid into cholesteryl ester. In contrast to the linear time course of apoA-I-mediated cholesterol release (Fig. 1C), the cholesterol pool rapidly decreased within the initial few hours after exposing the cells to apoA-I in WI-38 (**Fig. 2**), consistent with our previous reports (33, 34). Decrease of the ACAT-accessible cholesterol pool by apoA-I was also shown in BALB/3T3 (a mouse fibroblast cell line) and MRC-5 (a human fibroblast cell line) (by 17% and 24%, respectively, from the control at the 3 h incubation time with apoA-I),

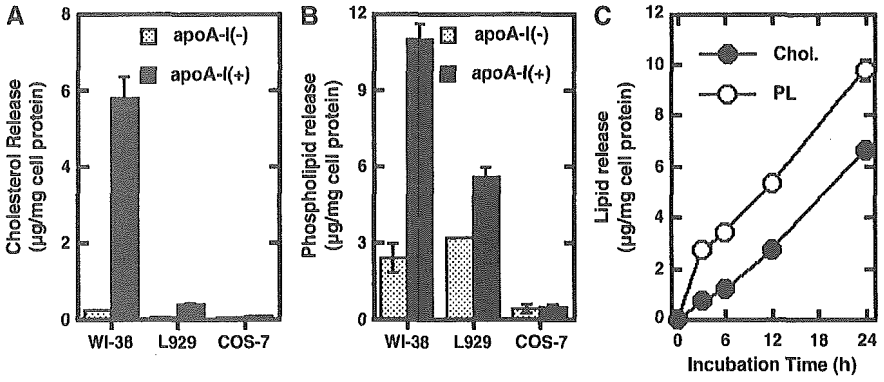

Fig. 1. Release of cellular lipid by apolipoprotein A-I (apoA-I) in fibroblast cell lines. WI-38, L929, and COS-7 cells were incubated with (+) and without (−) 10 μg/ml apoA-I for 24 h, and cholesterol (A) and choline-phospholipid in the medium (B) were measured. The time course of apoA-I-mediated cholesterol and phospholipid release in WI-38 cells is shown in C. Cholesterol (Chol.) and choline-phospholipid (PL) were measured enzymatically as described in Materials and Methods. The data represent mean ± SD of triplicate assays.

TABLE 2. Characterization of apoA-I-mediated reactions in the cells examined

Variable	WI-38	L929	COS-7
Cholesterol release by apoA-I	+	−	−
PL release by apoA-I	+	+	−
HDL generation[a]	+	+	−
FC/PL in the HDL[b]	0.65	0.14	NA[c]
ABCA1 expression[a]	+	+	−
Caveolin-1 expression[a]	+	+	+
Cholesterol translocation by apoA-I	+	−	−
ABCA1 increase by apoA-I	+	±	NA
PKC activation by apoA-I	+	−	−
Cholesterol translocation by PMA	+	+	−
ABCA1 increase by PMA	+	−	NA
PKC activation by PMA	+	+	+

apoA-I, apolipoprotein A-I; FC, free cholesterol; PKC, protein kinase C; PL, phospholipid; PMA, phorbol 12-myristate-13-acetate.

[a]From ref. (16)

[b]Determined from the results shown in Fig. 1.

[c]NA, not applicable.

both of which generate cholesterol-rich HDL in the presence of apoA-I. In contrast, no change of the ACAT-accessible cholesterol pool size was observed in L929 cells or in COS-7 cells that generate cholesterol-poor HDL or no HDL by apoA-I, respectively (Fig. 2).

To confirm a relationship between the ABCA1-mediated cholesterol release by apoA-I and the decrease of the ACAT-accessible cholesterol pool, HEK293 cells stably expressing human ABCA1 (293/2c) were compared with nontransfected HEK293 cells. Wild-type HEK293 did not express ABCA1 at a detectable level by Western blotting (23) and released neither phospholipid nor cholesterol by apoA-I (**Fig. 3A, B**). ApoA-I also failed to reduce the ACAT-accessible cholesterol pool in the cells (Fig. 3C). In contrast, 293/2c cells demonstrated an apoA-I-mediated release of cholesterol and phospholipid and a decrease of ACAT-accessible cholesterol by apoA-I (Fig. 3). It is thus clear that apoA-I reduces the ACAT-accessible pool as it mediates cholesterol removal in the presence of ABCA1 activity.

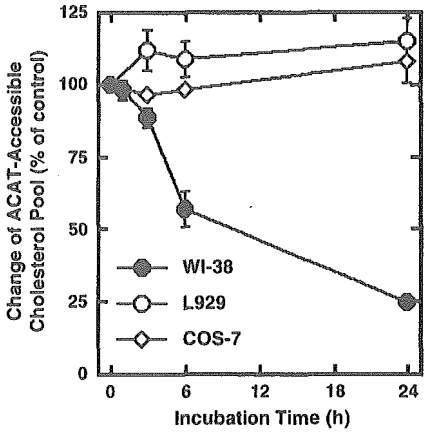

Fig. 2. Change of the ACAT-accessible cholesterol pool by apoA-I. Cells were incubated with or without 10 μg/ml apoA-I for the indicated times, and 1.5 μCi/ml [^{14}C]oleic acid was included during the final 1 h for measurement of its incorporation into cholesteryl ester. Data represent means ± SD for percentage of control (incubation without apoA-I) based on percentage of cholesteryl [^{14}C]oleate to the total cellular incorporation of [^{14}C]oleic acid. The control values were all of approximately the same order of magnitude, such as 4×10^4 dpm/mg cell protein for WI-38, 3×10^4 dpm/mg for COS-7, and 4×10^4 dpm/mg for L929.

Effect of ACAT enzyme activity on apoA-I-mediated cholesterol release

To examine the role of the ACAT enzyme in the regulation of the cholesterol pool available for ABCA1-mediated cholesterol release by apoA-I, we treated cells with a potent and specific ACAT inhibitor. Treatment of the CHO cell-derived clone, 25RA, with an ACAT inhibitor, F12511, resulted in a substantial increase in apoA-I-mediated cholesterol release (**Fig. 4A**). It also caused an increase in phospholipid release, although smaller than the cholesterol increase (Fig. 4A). Treating cells with the ACAT inhibitor F12511 caused ABCA1 upregulation (Fig. 4C), consistent with our previous finding in mouse peritoneal macrophages using a different ACAT inhibitor (35). To avoid the use of ACAT inhibitors that may cause nonspecific side effect(s), we examined ACAT-1-deficient cells, AC29 (25), and AC29 stably expressing human ACAT-1 (AC29/hACAT1) were also examined to assess the role of ACAT. Table 1 shows cellular cholesterol and ACAT activity in these CHO mutants. ApoA-I-mediated cholesterol release was 9-fold higher in the AC29 cell than its parental cell, 25RA (Fig. 4B). Expression of human ACAT-1 in AC29 cells partially reversed the apoA-I-mediated cholesterol release, although it was still higher than that in 25RA cells, presumably because of the higher free cholesterol level in AC29/hACAT1 cells than in 25RA cells (Table 1). Phospholipid release by apoA-I was also enhanced in AC29 cells, although not as much as the cholesterol release; phospholipid release was slightly higher in AC29/hACAT1 than in 25RA cells (Fig. 4B). We next examined the cellular ABCA1 levels in these cells treated with or without apoA-I. ABCA1 levels in these mutants were counterregulated by the expression of ACAT, as shown in Fig. 4C. ApoA-I further increased ABCA1 even in the ACAT-deficient cells (Fig. 4D). These results suggested that the mechanisms that cause the increase in ABCA1 protein content by inactivation of ACAT and by exposure to apoA-I are different. Inactivation of ACAT may cause an increase of ABCA1 expression as a result of transcription activation (35), whereas lipid-free apolipoprotein stabilizes the cellular ABCA1 protein against degradation (32). Irrespective of the mechanisms involved, change in ABCA1 expression seems correlate with the increase of apoA-I-mediated phospholipid release rather than cholesterol release (Fig. 4).

PKC activation induces the translocation of intracellular cholesterol from the ACAT-accessible pool for apoA-I-mediated release

It has been reported that PKC is involved in the change of the ACAT-accessible cholesterol pool in rat vascular

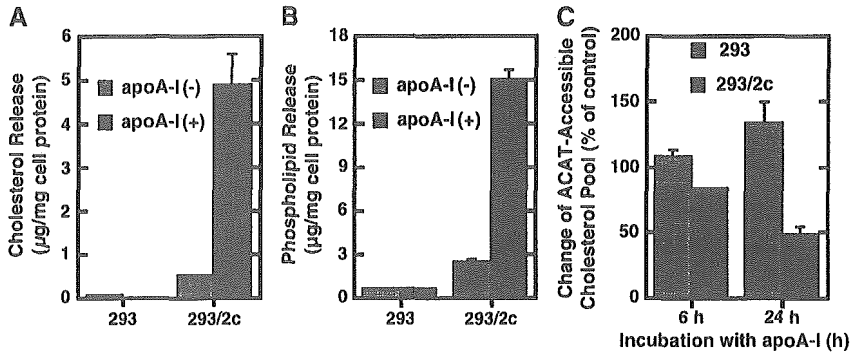

Fig. 3. ABCA1 requirement for apoA-I-induced intracellular cholesterol translocation. apoA-I-mediated cholesterol (A) and phospholipid (B) release and change of the ACAT-accessible cholesterol pool by apoA-I (C) in ABCA1-transfected (293/2c) and wild-type HEK293 (293) cells were examined. A, B: apoA-I-mediated cholesterol and phospholipid release were determined enzymatically after incubation of cells with and without apoA-I (10 μg/ml) for 24 h. C: Change of the ACAT-accessible cholesterol pool was assayed by incorporation of 1.0 μCi/ml [^{14}C]oleic acid into cholesteryl ester as described for Fig. 2. The data represent means ± SD of triplicate determinations as expressed as percentage of control (without apoA-I).

smooth muscle cells and mouse peritoneal macrophages (33, 34). To extend these early studies, we treated various cell types with PKC activators to monitor the effect of the change in the ACAT-accessible cholesterol pool. The change of the cholesterol pool was demonstrated with the short-term treatment of cells with 160 nM PMA, which leads to PKC activation (**Fig. 5A**). WI-38 and L929 showed decreases of this cholesterol pool by PKC activation in the absence of cholesterol acceptor, whereas a reduction was not observed in COS-7, in which ABCA1 expression is not detected. Treatment with DOG, another PKC activator, also induced the reduction of the ACAT-accessible cholesterol pool in WI-38 (data not shown). In contrast, 4α-PMA, a control compound of PMA that possesses no stimulating effect on PKC, had no effect on the reduction of the ACAT-accessible cholesterol pool in WI-38 (data not shown). Thus, PKC seems to trigger cholesterol translocation from the ACAT-accessible pool.

PKC activation by apoA-I was previously demonstrated in WI-38 human fibroblasts (24). We examined whether apoA-I can also activate PKC in L929 and COS-7 cells. Both cells were treated with apoA-I for 5–120 min, and the membrane-associated PKC activities were then measured. We found no PKC activation by apoA-I at any point during this time course in these cell lines. The control experiment showed that a 20 min PMA treatment increased membrane-associated PKC activity in both cell lines: 1.6-fold in COS-7 cells and 2.1-fold in L929 cells. Thus, apoA-I failed in the activation of PKC, reduction of the ACAT-accessible cholesterol pool, and induction of cholesterol release in L929, whereas pharmacological activation of PKC induced the reduction of this cholesterol compartment. Therefore, we examined the effect of PMA on the apoA-I-mediated cholesterol release in this cell line (Fig. 5B). L929 cells were pretreated with 160 nM PMA for 30 or 60 min before incubation with apoA-I. A significant increase by PMA treatment was observed in the apoA-I-mediated release of cholesterol ($P < 0.05$, Student's t-test) when measured as the short-term release of [^{3}H]cholesterol (Fig. 5B), although it was still poor and no measurable mass was detected in the medium because it was still under the detection limit of the assay method. The apoA-I-dependent cholesterol release was increased by ~2.7 times. The release of phosphatidylcholine and sphingomyelin by apoA-I was not influenced by PMA, resulting in cholesterol "enrichment" in the conditioned medium. Thus, PKC activation induced intracellular cholesterol translocation from the ACAT-accessible pool, presumably to the site for the apoA-I/ABCA1-mediated release in L929 cells.

Change in ABCA1 protein level by apoA-I and PMA

We examined the effect of apoA-I and PMA on change in ABCA1 expression level. As we reported (24), apoA-I treatment resulted in an increase of ABCA1 in WI-38 as a result of the retardation of proteolytic degradation (**Fig. 6A**). On the other hand, apoA-I failed to increase ABCA1 protein in L929 within 4 h (Fig. 6A), although a longer incubation (24 h) increased it to some extent (data not shown). The PMA treatment that leads to PKC activation resulted in an increase of ABCA1 by 1.4-fold in WI-38 cells in 1 h, consistent with our previous report (24), whereas the same treatment did not affect ABCA1 expression in L929 (Fig. 6B). Therefore, the stimulations that lead to ABCA1 stabilization in WI-38 human fibroblasts were inefficient in L929 cells.

DISCUSSION

We have suggested differential regulation of cholesterol release and phospholipid release in the apolipoprotein/ABCA1 pathway to generate HDL, based on the following observations. Cholesterol contents in the HDL generated by the apolipoprotein-cell interaction is cell specific (16,

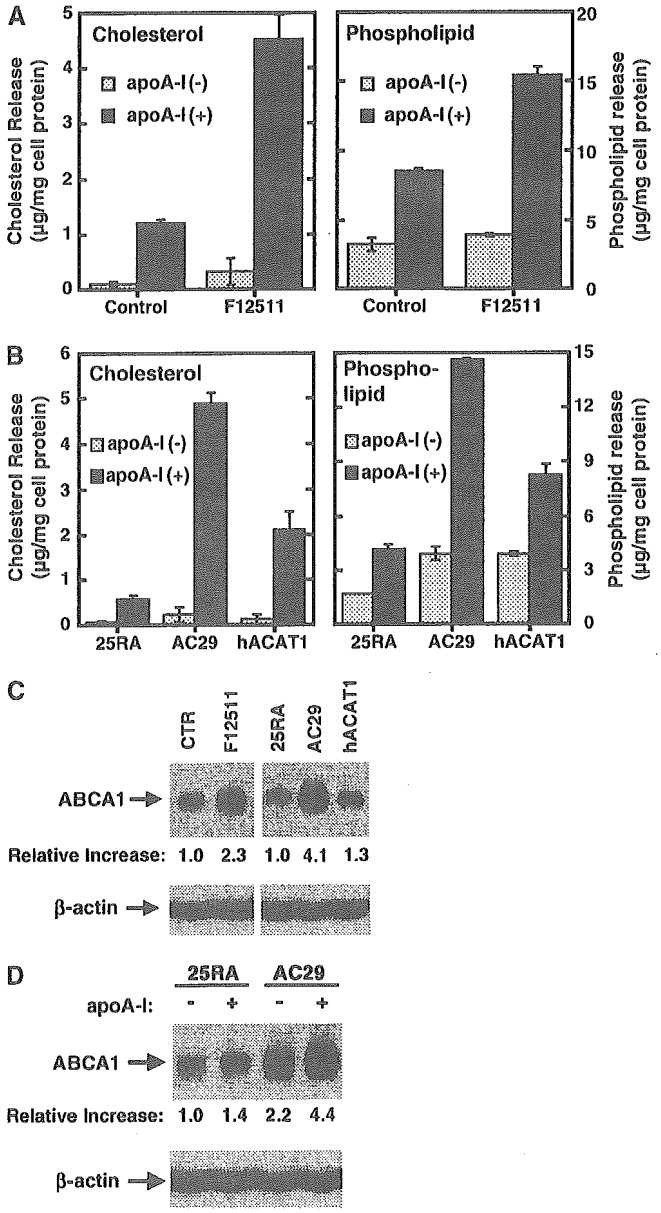

Fig. 4. Effect of ACAT activity on apoA-I-mediated cholesterol release and cellular ABCA1 levels. A: 25RA cells were seeded into six-well trays at a density of 1.5×10^5 cells/well and were grown for 3 days. The cells were incubated with (+) and without (−) 5 μg/ml apoA-I in the presence or absence of the ACAT inhibitor F12511 (400 nM) for 24 h, and release of cholesterol and choline-phospholipid were measured. The data represent means ± SD in triplicate assays. B: apoA-I-mediated release of cholesterol and phospholipid from ACAT-deficient cells. The CHO mutant, 25RA, its ACAT-deficient mutant, AC29, and human ACAT-1-expressing AC29 cells, AC29/hACAT1, were grown as described above. The cells were then incubated with (+) and without (−) 5 μg/ml apoA-I for 24 h, and releases of cholesterol and choline-phospholipid were measured. The data represent means ± SD of the apoA-I-dependent lipid release in triplicate assays. C, D: Cellular ABCA1 protein level was examined in CHO mutant cells. CHO cells (25RA) were incubated with and without an ACAT inhibitor, F12511 (400 nM) [F12511 and CTR (control), respectively] for 24 h in the medium containing 10% FBS (C, left panel). The right panel of C shows the results with 25RA, its ACAT-deficient mutant AC29, and AC29 transfected with ACAT-1 (AC29/hACAT1) under the same incubation conditions without an ACAT inhibitor. The effect of apoA-I on ABCA1 levels was examined for 25RA cells and AC29 cells as incubated with (+) and without (−) 5 μg/ml apoA-I for 24 h in 0.1% BSA. Equal amounts of whole cell lysate protein (80 μg protein/lane) from the cells indicated were subjected to immunoblot analysis using anti-ABCA1 antibody or anti-β-actin antibody as a loading control. The signal intensity of ABCA1 was measured as described in Materials and Methods, and relative increases of ABCA1 are indicated. The data represent mean values of two or three separate scanning results, and similar results were obtained in two separate experiments. Expression of β-actin did not change between the cells compared.

36). Apolipoprotein-mediated cellular cholesterol release was accompanied by a rapid reduction of the intracellular pool of cholesterol available to ACAT within the initial few hours in mouse peritoneal macrophages, whereas cholesterol release was linear for at least 24 h (33). PKC inhibitors and activators modulated both cholesterol content in the HDL generated by the apolipoprotein-cell interaction and change in the ACAT-accessible cholesterol pool in certain cells under certain conditions (33, 34). More recent studies have shown that caveolin-1 is involved in cholesterol enrichment of the HDL generated by apoA-I-mediated lipid release in THP-1 cells (10) and that plasma membrane lipid composition modulates apoA-I/ABCA1-mediated cholesterol release but not phospholipid release (37). On the other hand, pharmacological inhibition of ACAT increased ABCA1 through the enhancement of its transcription (35). Thus, in the current work, we attempted to establish a role of ACAT in the apoA-I/ABCA1-mediated HDL assembly and investigated potential factors involved in the mobilization of intracellular cholesterol for HDL assembly.

Table 2 summarizes the apoA-I-mediated reactions in the fibroblasts examined. ABCA1 was expressed in WI-38 and L929 (24). Consequently, HDL was generated by apoA-I with WI-38 and L929, but no HDL was produced with COS-7. However, HDL produced with L929 contained almost no cholesterol (16). ApoA-I induced the reduction of the ACAT-accessible cholesterol pool in WI-38 cells but not in COS-7 or L929, neither of which exhibits cholesterol re-

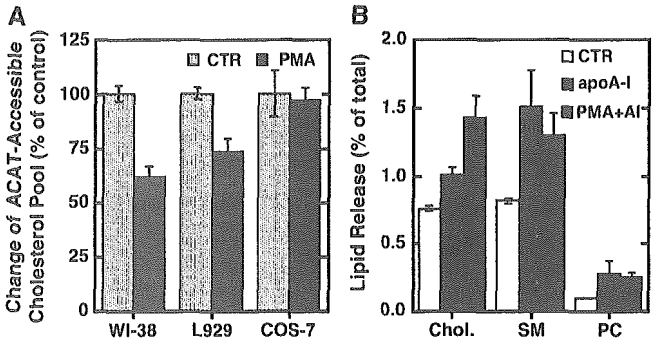

Fig. 5. Effect of phorbol 12-myristate-13-acetate (PMA) on the ACAT-accessible cholesterol pool and apoA-I-mediated cholesterol release in L929. A: Cells grown in six-well trays were stimulated with 160 nM PMA in the presence of 1.5 μCi/ml [^{14}C]oleic acid for 1 h, and incorporation of the radioactivity into cholesterol ester was measured as in Materials and Methods. The data points represent means ± SD of triplicate assays. CTR, control. B: L929 cells were incubated with 5 μCi/ml [^{3}H]cholesterol or 5 μCi/ml [^{3}H]choline chloride for 24 h as described in Materials and Methods. The cells were treated with or without 160 nM PMA for 30 min. Cells were washed three times with PBS followed by incubation of the cells with or without 10 μg/ml apoA-I for 3 h. Radiolabeled cholesterol (Chol.), phosphatidylcholine (PC), and sphingomyelin (SM) in the medium and cells were determined by TLC. The data represent means ± SD of triplicate assays expressed as percentage of release of the respective lipid.

lease by apoA-I. The reduction of the ACAT-accessible cholesterol pool by apoA-I was also observed in HEK293 stably expressing ABCA1 but not in wild-type HEK293 cells. Thus, the reduction of this compartment is related to the release of cholesterol by the apoA-I/ABCA1 reaction but not directly to ABCA1 expression and the generation of HDL with cellular phospholipid. These results indicate that cholesterol is mobilized from the ACAT-accessible pool for cholesterol enrichment of the HDL to be generated by the apolipoprotein/ABCA1-mediated reaction. Inactivation of ACAT-1 resulted in increases in both ABCA1 expression and lipid release, but the increase in ABCA1 expression related more directly to the apoA-I-mediated phospholipid release than did the cholesterol release. The change in cholesterol release by apoA-I was almost twice as great as the changes in phospholipid release and ABCA1 expression. These results are consistent with the finding of an increase of HDL-cholesterol in ACAT-1-deficient mice (38). We thus propose that ACAT-1 enzyme activity directly modulates the ABCA1/apolipoprotein-mediated HDL assembly by regulating both ABCA1 expression and the mobilization of cellular cholesterol.

As mentioned above, PKC activity seems to modulate the ACAT-accessible cholesterol pool (33, 34). For further characterization of this phenomenon, various fibroblast cells were treated with a PKC activator. Direct activation of PKC by PMA induced a reduction of the ACAT-accessible cholesterol pool in most of the cell types that produce cholesterol-rich HDL. Interestingly, PMA decreased the ACAT-accessible cholesterol in L929 cells. In these cells, apoA-I produced cholesterol-poor HDL but failed to reduce the ACAT-accessible cholesterol compartment. Accordingly, HDL produced from the PMA-treated L929 was relatively "enriched" with cholesterol. Therefore, PKC activation seems to trigger cellular cholesterol mobilization.

It remains to be investigated how apoA-I and/or PKC stimulates intracellular cholesterol transport. Relevant to this question is the finding that phosphorylation of caveolin-1 at serine 80 may modulate its cholesterol binding and apoA-I-mediated cholesterol release (39). Vesicular transport is also a focus of the study of cholesterol trafficking. ABCA1 is localized in intracellular compartments such as endosomes and the Golgi (13, 40). ApoA-I stimulates vesicular transport from the Golgi to the plasma membrane (41), and transport of lipids from the Golgi to

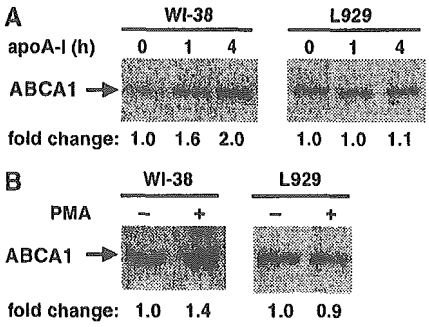

Fig. 6. Change of ABCA1 protein by apoA-I or PMA in WI-38 and L929 fibroblast cells. Cells were incubated with apoA-I (10 μg/mL) for the indicated times (A) or with 320 nM PMA for 1 h (B), and the membrane fraction was then prepared. Equal amounts of membrane protein (100 μg/lane for WI-38 and 200 μg/lane for L929) were subjected to immunoblotting using anti-ABCA1 antibody. The signal intensity for ABCA1 was measured as described in Materials and Methods, and relative changes of ABCA1 are shown. The data represent means of two or three scanning results, and similar results were obtained in two separate experiments. Consistency of the protein loading was verified by Coomassie Brilliant Blue staining of the gels (data not shown).

the plasma membrane is defective in the ABCA1-deficient cells (13). In addition, ABCA1 is reportedly involved in late-endosome vesicular trafficking (42). However, none of these reports directly indicate the involvement of PKC in the modulation of vesicular transport or ABCA1 localization.

ABCA1 is protected from calpain-mediated proteolytic degradation in the presence of lipid-free apolipoprotein (32, 43). We have recently demonstrated that apoA-I activates PKCα to phosphorylate and stabilize ABCA1 (24). In that study, we found a greater effect of PKC inhibitors on apoA-I-mediated cholesterol release when the inhibitors prevented both cholesterol and phospholipid release (24). In the current paper, we demonstrate that PKC also plays a role in the intracellular translocation of cholesterol for ABCA1-mediated HDL assembly by apoA-I. Thus, these dual effects of PKC activation may account for the difference of the inhibitory effect of PKC inhibitors on apoA-I-mediated cholesterol and phospholipid release.

However, apoA-I and PMA both failed to increase ABCA1 in L929 mouse fibroblast cells, inconsistent with our previous reports showing that release of phospholipid, presumably sphingomyelin, induces PKC activation by phosphatidylcholine-specific phospholipase C-mediated diacylglycerol production, leading to phosphorylation and stabilization of ABCA1 in WI-38 human fibroblasts (24). Another mouse fibroblast cell line, BALB/3T3, and mouse peritoneal macrophages both showed very poor increases of ABCA1 by apoA-I (16, 35). In addition, PKC inhibitors prevented only apoA-I-mediated cholesterol release in mouse macrophages (34). These results may indicate insufficiency of the PKC signaling pathway to regulate ABCA1 stabilization in murine cells.

In summary, the results in this report fit the conclusion that PKC plays a role in the apolipoprotein/ABCA1-mediated cholesterol release by inducing not only ABCA1 phosphorylation and stabilization but also intracellular cholesterol mobilization for its release, at least in human cells. ApoA-I mobilizes intracellular cholesterol from the ACAT-accessible compartment for ABCA1-mediated release via a process involving PKC signaling. In addition, ACAT-1 directly controls cholesterol availability for ABCA1-mediated release.

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