

On the hepatic mechanism of HDL assembly by the ABCA1/apoA-I pathway

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Abstract The mechanism for the assembly of HDL with cellular lipid by ABCA1 and helical apolipoprotein was investigated in hepatocytes. Both HepG2 cells and mouse primary culture hepatocytes produced HDL with apolipoprotein A-I (apoA-I) whether endogenously synthesized or exogenously provided. Probucol, an ABCA1 inactivator, inhibited these reactions, as well as the reversible binding of apoA-I to HepG2. Primary cultured hepatocytes of ABCA1-deficient mice also lacked HDL production regardless of the presence of exogenous apoA-I. HepG2 cells secreted apoA-I into the medium even when ABCA1 was inactivated by probucol, but it was all in a free form as HDL production was inhibited. When a lipid-free apoA-I-specific monoclonal antibody, 725-1E2, was present in the culture medium, production of HDL was suppressed, whether with endogenous or exogenously added apoA-I, and the antibody did not influence HDL already produced by HepG2 cells. **¶** We conclude that the main mechanism for HDL assembly by endogenous apoA-I in HepG2 cells is an autocrine-like reaction in which apoA-I is secreted and then interacts with cellular ABCA1 to generate HDL.—Tsujita, M., C-A. Wu, S. Abe-Dohmae, S. Usui, M. Okazaki, and S. Yokoyama. **On the hepatic mechanism of HDL assembly by the ABCA1/apoA-I pathway.** *J. Lipid Res.* 2005. 46: 154–162.

Supplementary key words cholesterol • high density lipoprotein • hepatocytes • HepG2 • probucol • apolipoprotein A-I • ATP binding cassette transporter A1

High density lipoprotein is produced by the reaction of helical apolipoprotein and ABCA1 (1). This is considered a main source of plasma HDL, because familial HDL deficiency (Tangier disease) has been identified as the defect of HDL assembly by this reaction (2) caused by the mutation of ABCA1 (3–5). The ABCA1/apolipoprotein reaction is also an important pathway of cellular cholesterol release for its conversion to bile acids in the liver, along

with an alternative nonspecific diffusion pathway accelerated by cholesterol esterification with LCAT on HDL particles (1). The main site for HDL production is generally thought to be the liver and intestine, where the cells synthesize helical apolipoprotein, mainly apolipoprotein A-I (apoA-I), and produce HDL, presumably upon the interaction of this apolipoprotein with its own ABCA1 by removing cellular lipid (6–8). However, it is unclear in which step of the apoA-I production and secretion this reaction takes place for the assembly of HDL. HDL particles have never been clearly identified in the secretory pathway of any HDL-producing cell, including hepatocytes.

Probucol is an inhibitor of apoA-I-mediated cellular cholesterol release and HDL assembly (9, 10) and has been identified as an inactivator of ABCA1 (11). We used this compound in LCAT-deficient mice in attempting to suppress the two major cholesterol-release pathways of somatic cells described above (12). To our surprise, no systemic cholesterol accumulation was observed, indicating that cholesterol may leave cells by a nonspecific pathway and that many extracellular acceptors can act as cholesterol transporters, such as albumin and blood cells. However, cholesterol content increased only in the liver when probucol inhibited the ABCA1 pathway in the cholesterol-fed LCAT-deficient mice. Thus, the liver seems to be a major organ from which cholesterol release requires the ABCA1 pathway, and these results indicate that the liver is a major source of plasma HDL (13), consistent with other reports using genetically engineered animals (6–8).

It is thus important to characterize how HDL is assembled in hepatocytes with helical apolipoproteins synthesized by hepatocytes interacting with their own ABCA1. We used the human hepatoma cell line HepG2 and mouse primary culture hepatocytes as model systems. To identify

Abbreviations: apoA-I, apolipoprotein A-I; FCS, fetal calf serum; MEM- α , minimum essential medium Eagle α modification.

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a role of ABCA1 in HDL production by hepatocytes, we used probucol to inactivate ABCA1 as well as ABCA1-deficient mice. A monoclonal antibody specific against lipid-free apoA-I was used as a tool to trap lipid-free apoA-I to examine whether apoA-I is secreted from the cells as a free form before it interacts with ABCA1 of the cells to generate HDL.

MATERIALS AND METHODS

Apolipoprotein, lipoprotein, and an anti-apoA-I monoclonal antibody

ApoA-I and apoA-II were purified from human HDL fraction using delipidation and anion-exchange column chromatography in 6 M urea as previously described (13, 14). Apolipoproteins were dissolved in 50 mM sodium phosphate buffer, pH 7.4, containing 6 M guanidine-HCl and thoroughly dialyzed against 10 mM sodium phosphate buffer, pH 7.4, 0.15 M NaCl (PBS). For the specific binding study, apoA-I was labeled with ^{125}I as previously described (9) except for the use of Iodo-Beads to activate ^{125}I . The iodine-labeled apoA-I was concentrated by a Ultrafree-15 centrifugal filter device (Millipore Corp.). One milliliter of ^{125}I -apoA-I

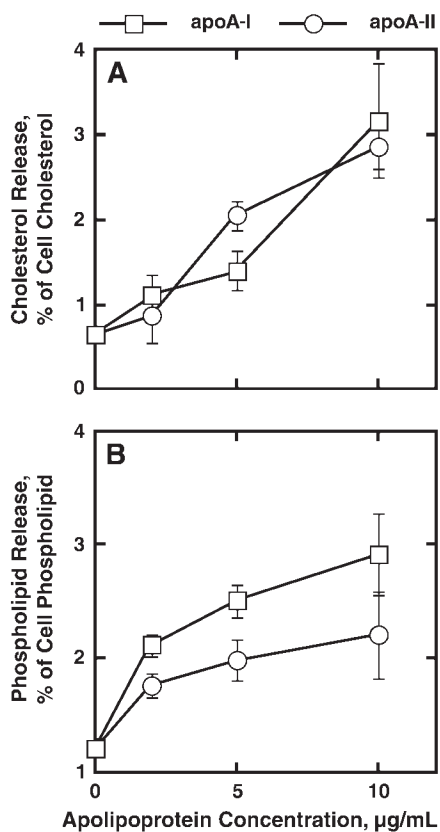


Fig. 1. Apolipoprotein-mediated lipid release from HepG2 cell. Cells were incubated for 16 h with minimum essential medium Eagle α modification (MEM- α) containing 0.02% BSA and the indicated concentration of apolipoprotein A-I (apoA-I) or apoA-II. Cellular lipid released into the conditioned medium was extracted by organic solvent and analyzed by colorimetric enzymatic assay for cholesterol and choline-phospholipid as described. The values represent means \pm SEM for three determinations. Open circles, exogenous human apoA-I; open squares, exogenous human apoA-II.

solution was dissolved in an equal volume of 6 M guanidine-HCl in 50 mM sodium phosphate buffer, pH 7.4, and dialyzed against PBS to remove unbound ^{125}I and guanidine-HCl. Probucol was kindly provided by Daiichi Pharmaceutical Co. LDL was isolated by sequential ultracentrifugation, and control and probucol-containing LDL were prepared by the method previously described (9). A monoclonal antibody (IgG) against lipid-free apoA-I, 725-1E2, was among the antibodies provided by Daiichi Pure Chemicals (Tokyo, Japan) and characterized in our laboratory as described previously (15). Mouse IgG was purchased from Chemicon International and used as a nonspecific control for 725-1E2.

HepG2 cells

HepG2 cells (American Type Culture Collection; ATCC HB8065) were maintained in minimum essential medium Eagle α modification (MEM- α) supplemented with 10% fetal calf serum (FCS) and antibiotics (5 U/ml penicillin and 5 $\mu\text{g}/\text{ml}$ streptomycin).

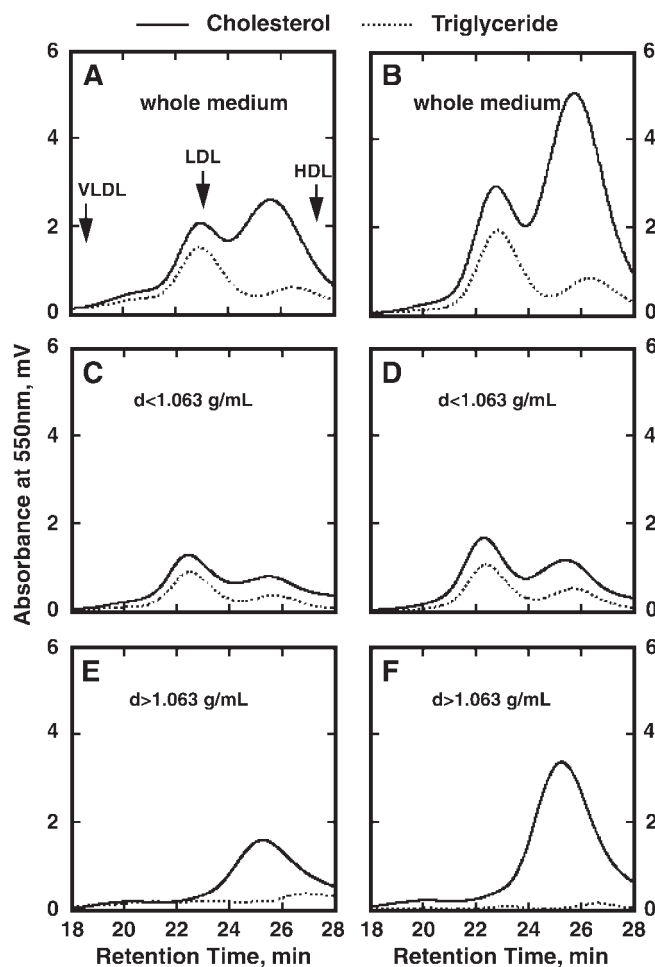


Fig. 2. Lipoprotein analysis of the culture medium of HepG2 cells by HPLC. Cells were incubated with MEM- α containing 0.02% BSA with (B, D, F) or without (A, C, E) 10 $\mu\text{g}/\text{ml}$ human apoA-I for 16 h. The conditioned medium of HepG2 cells (100 μl) was analyzed by the HPLC lipoprotein analysis system using two tandem gel-permeation columns (Lipopropak XL; 7.8 mm \times 300 mm; Tosoh). The elution profile was monitored by an online assay system for total cholesterol (solid lines) and triacylglycerol (dotted lines). A and B: Whole conditioned medium. C and D: $d > 1.063$ g/ml fraction. E and F: $d < 1.063$ g/ml fraction. The eluting positions of human plasma VLDL, LDL, and HDL are indicated by arrows in A.

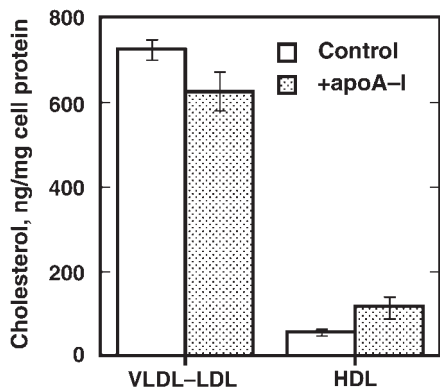


Fig. 3. Analysis of lipoprotein generated by mouse primary culture hepatocytes. Primary culture hepatocytes of C57Bl/6 mice were harvested using the Hanks'-EDTA and collagenase two-medium method described in Materials and Methods. The cells were incubated in DMEM (high glucose)/BSA medium with or without human apoA-I for 16 h. The conditioned medium was separated by ultracentrifugation at a density 1.063 g/ml. The data represent means \pm SEM of three determinations. Difference in HDL between control and apoA-I (+) is significant by $P < 0.005$.

For the individual experiments, cells were subcultured onto 35 or 60 mm plates at the density of 0.7 to 1×10^6 cells/ml and maintained in MEM- α with 10% FCS and antibiotics (5 U/ml penicillin and 5 μ g/ml streptomycin) by changing the medium every 2 days. On the fifth day, when the cells were 80–90% confluent, the cells were washed extensively with MEM- α and incubated with MEM- α containing 0.02% BSA and antibiotics with and without exogenous human apoA-I and human apoA-II for 16 h. The conditioned medium was collected for further lipid analysis. To load probucol, cells at 65–75% confluence were incubated with LDL or probucol-containing LDL (50 μ g/ml as protein) in MEM- α for 24 h before the lipid-release assay.

Mouse primary hepatocytes

C57Bl/6 mice (9 weeks old) were purchased from the local experimental animal supplier. The mice were fed with normal mouse chow or chow containing 0.5% (w/w) probucol ad libitum. ABCA1-deficient mice were bred from ABCA1 heterozygote mice (DBA/1-Abca1^{tm1Jdm}/J) purchased from Jackson's Animal Laboratories (Stony Brook, NY). The genotypes of all offspring were determined by PCR analysis of tail DNA. The oligonucleotide primer sets 5'-TGGGAAGCTCTGCTAAAAT-3', 5'-CCATGTGGTGTGTAGACA-3' and 5'-TTTCTCATAGGGTTGGTCA-3', 5'-TGCAATCCATCTTGTTCAT-3' were used to determine the wild-type and mutant alleles, respectively. PCR was performed according to the genotyping method provided by the animal supplier except for an annealing temperature of 58°C. The mouse

primary hepatocytes were harvested and cultured according to the method by Noga et al. (16). Mice were anesthetized and the liver was perfused with Hanks' salt solution without calcium containing 0.5 mM EGTA and Hanks' salt solution with calcium and magnesium containing 75 U/ml collagenase (type IV) at 37°C. The hepatocytes were isolated by low-gravity centrifugation and placed onto sterilized collagen-coated 60 mm culture dishes (1.8×10^6 cells/dish). After 2 h, when cells were attached as a monolayer, the unbound cells were washed with DMEM (high glucose) containing 0.02% BSA. The cells were incubated with and without apoA-I for 16 h, and the conditioned medium was collected after the incubation. All of the experiments were completed within 24 h after harvesting the cells. The experimental procedure had been approved by the Animal Welfare Committee of Nagoya City University Graduate School of Medical Sciences according to institutional guidelines.

Lipoprotein analysis in the conditioned medium

Lipoprotein in the conditioned medium of the hepatocytes was analyzed by HPLC using a gel-permeation column(s) (Lipopropak XL; 7.8 mm \times 300 mm; Tosoh) with 0.05 M Tris-buffered acetate, pH 8.0, containing 0.3 M sodium acetate, 0.05% sodium azide, and 0.005% Brij-35 at a flow rate of 0.7 ml/min and an on-line enzymatic lipid-detection system (10, 17–19). The conditioned medium was centrifuged at 10,000 rpm for 5 min to remove cell debris, and a 200 μ l aliquot was applied for HPLC analysis. The method was thoroughly validated against the reference methods of ultracentrifugation and of Superose gel-permeation chromatography, including the criteria of subfraction analysis of HDL (17, 19). In some experiments, the VLDL/LDL fraction ($d < 1.063$ g/ml) and the HDL fraction ($1.063 < d < 1.21$ g/ml) were isolated from the conditioned medium by sequential ultracentrifugation at 1.063 g/ml and 1.21 g/ml in a Hitachi CS120GX (Hitachi) at 99,000 rpm for 4 h. Lipid was extracted from the total conditioned medium and the VLDL/LDL and HDL fractions with organic solvent, and cholesterol and choline-phospholipid were determined with colorimetric enzyme assay kits (Kyowa Medics Co., Ltd., for cholesterol and Wako Pure Chemical Industries, Ltd., for phospholipid) (20). Electrophoretic analysis was also performed for the medium using a Beckman Paragon System on an agarose gel.

Apolipoprotein analysis

Apolipoproteins in the HepG2 conditioned medium were analyzed by immunoblotting using rabbit antiserum raised against human apoA-I and goat anti-human apoB IgG (affinity purified; Academy Bio-Medical Co., Inc.). The distribution of apoA-I and apoB in the HPLC-fractionated samples was analyzed. The eluent was fractionated every 30 s. After adding 4 μ g of BSA to each sample, protein was precipitated with 15% (w/v) trichloroacetic acid for 30 min on ice and recovered by centrifugation at 15,000 rpm for 10 min. The precipitated protein was washed with 1 ml

TABLE 1. Chemical compositions of cellular lipids in HepG2 cells

Sample	Total Cholesterol	Triglyceride	Phospholipid	Probucol
	μ g/mg protein			
Control LDL				
exo-apoA-I(-)	22.02 \pm 0.93	10.09 \pm 2.90	201 \pm 5.85	0.00
exo-apoA-I(+)	21.25 \pm 0.35	13.91 \pm 5.62	213 \pm 6.61	0.00
Probucol LDL				
exo-apoA-I(-)	19.84 \pm 0.63	15.12 \pm 1.72	169 \pm 10.45	1.01 \pm 0.21
exo-apoA-I(+)	21.17 \pm 0.84	19.08 \pm 2.48	185 \pm 8.60	1.17 \pm 0.27

exo-apoA-I, exogenous apolipoprotein A-I. Cellular lipid was analyzed by enzymatic methods, and probucol was measured using an HPLC method.

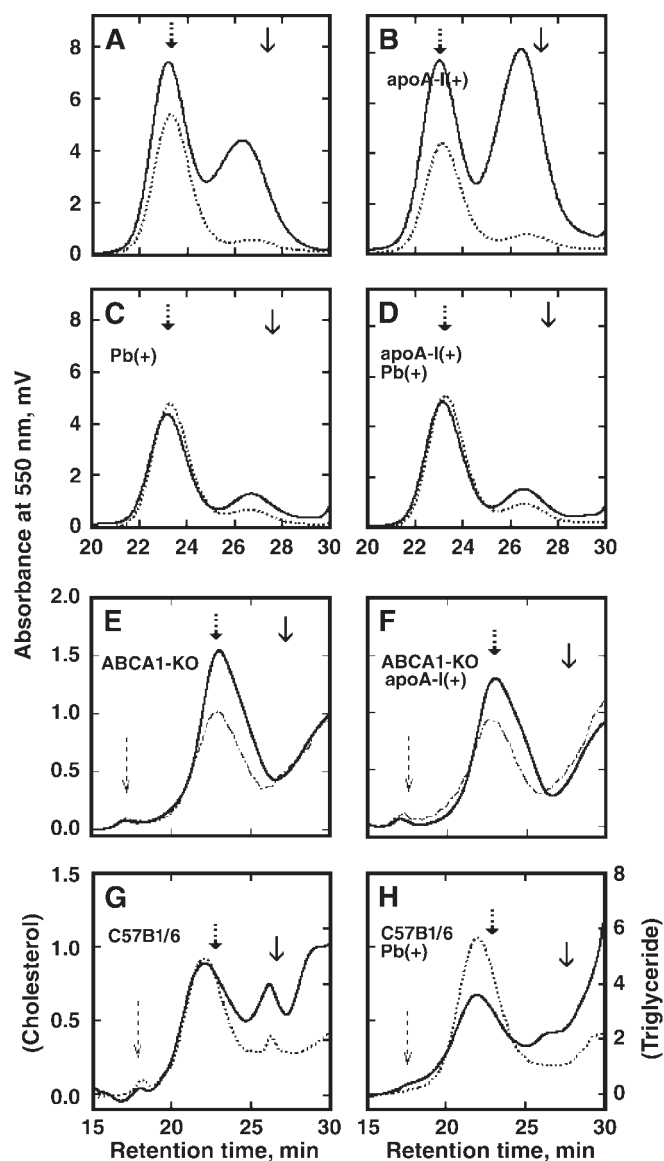


Fig. 4. The effect of ABCA1 inactivation on the generation of HDL by hepatocytes as analyzed by HPLC. A–D: HepG2 cells. A: The conditioned medium of HepG2 cells after 16 h. B: The conditioned medium incubated with apoA-I (10 $\mu\text{g}/\text{ml}$) for 16 h. C: The conditioned medium of probucol-loaded HepG2 cells after 16 h. D: The conditioned medium of probucol-loaded HepG2 cells with apoA-I (10 $\mu\text{g}/\text{ml}$) for 16 h. E–F: Primary cultured hepatocytes. E: The conditioned medium of the hepatocytes isolated from an ABCA1-deficient mouse after 16 h of incubation. F: The conditioned medium of the hepatocytes of an ABCA1-deficient mouse after incubation with apoA-I (10 $\mu\text{g}/\text{ml}$) for 16 h. G: The conditioned medium of the hepatocytes obtained from C57Bl/6 mice fed with control chow after 16 h of incubation. H: The conditioned medium of the hepatocytes obtained from C57Bl/6 mice fed with 0.5% probucol-containing chow. The medium was analyzed by HPLC using two tandem gel-permeation columns (Lipopropak XL; 7.8 mm \times 300 mm; Tosoh) to yield the lipoprotein profiles as described in the text. Solid lines represent cholesterol, dotted lines represent triglyceride, and dot-and-broken lines (in E and F) represent choline-phospholipid. Arrows with a solid line, a thick broken line, and a thin broken line indicate the eluting positions of plasma HDL, LDL, and VLDL, respectively. Pseudopeaks generated by the color of the medium caused baseline shifts in the analysis of the primary culture medium as the sensitivity of the monitoring lipid was increased. KO, knockout.

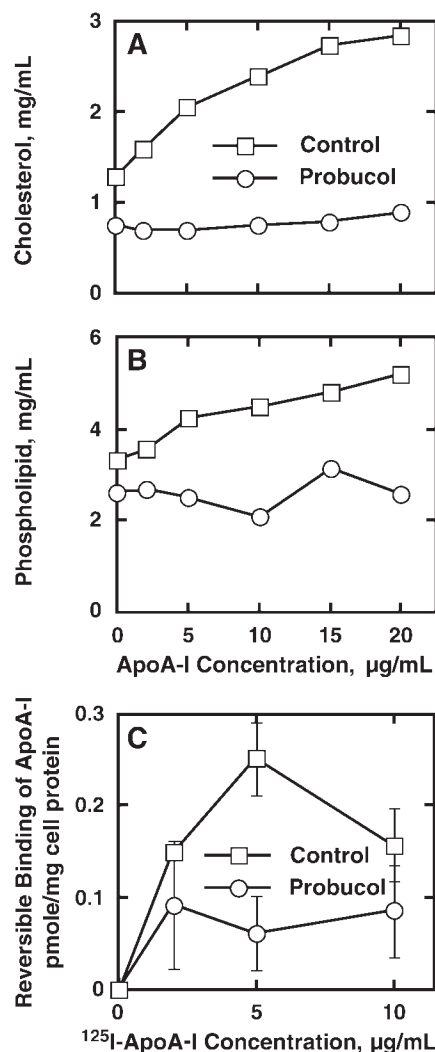


Fig. 5. Effect of probucol loading on apoA-I-mediated cellular lipid release from HepG2 cells and exchangeable binding apoA-I to HepG2 cells. A and B: Lipid release. HepG2 cells were incubated with either control LDL or probucol-containing LDL for 24 h. Cellular cholesterol and phospholipid release were measured after incubation for 16 h with various amounts of apoA-I. HDL-cholesterol (A) and HDL-phospholipid (B) were determined by an HPLC system. Open squares, control LDL-loaded cells; open circles, probucol-LDL loaded cells. C: ApoA-I binding. The indicated amounts of ^{125}I -apoA-I-incubated HepG2 cells were preloaded with control LDL or probucol-containing LDL for 2 h at 4°C. The cells were washed two times with ice-cold PBS, and the bound ^{125}I -apoA-I was displaced two times by 50 $\mu\text{g}/\text{ml}$ nonlabeled apoA-I for 9 h. The difference in the total radioactivity after the first washing and after the second displacement was calculated as displaceable or exchangeable binding of apoA-I to the cells. Squares, binding to the cells loaded with control LDL; circles, binding to the cells loaded with probucol-containing LDL. Error bars indicate \pm SEM.

of acetone and dissolved in 10 μl of sample buffer for SDS-polyacrylamide electrophoresis, incubated at 100°C for 5 min, and 5 μl of 9 M urea was added. The electrophoresis was performed on a polyacrylamide gel with a gradient of 4–20%. The conditioned media and fractionated media by ultracentrifugation were directly analyzed by immunoblotting against apoA-I. Anti-human albumin IgG fraction of rabbit antiserum was purchased from Sigma-Aldrich Inc. and used as a secondary antibody. Blot-

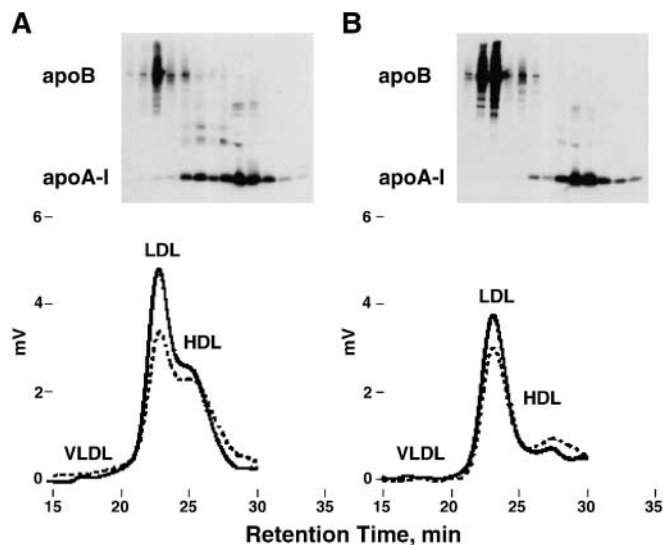


Fig. 6. Analysis of apolipoproteins in the HPLC fractions of the HepG2 conditioned medium. The conditioned medium of HepG2 cells (A) and probucol-loaded HepG2 cells (B) was tested after 16 h of incubation. The medium was analyzed by HPLC and fractionated as described in the text. The upper panels show immunoblot analysis of the fractions for apoB and apoA-I, and the lower panels represent HPLC elution profiles of lipids (solid lines, cholesterol; dotted lines, choline-phospholipid). Positions of lanes in the immunoblot analysis are adjusted for the HPLC elution times. VLDL, LDL, and HDL indicate the eluting positions for plasma lipoproteins.

ted bands were visualized by the chemiluminescence method (ECL Western Blotting Detection System; Amersham Pharmacia Biotech).

Clearance rate of ABCA1

The clearance rate of ABCA1 was analyzed by immunoblotting of the protein in the bulk cellular membrane fraction (21). HepG2 cells were loaded with control and probucol-containing LDL for 2 h and incubated in the presence of 71 μ M cycloheximide to inhibit the synthesis of new protein. The cells were harvested and applied for ABCA1 immunoblotting using a specific antibody raised against a C-terminal peptide of human ABCA1 (21).

Other methods

Cellular lipid was analyzed as described previously (20). The sample was also used for probucol analysis using an HPLC system (22). Protein was measured with the bicinchoninic acid method using BSA as a standard.

RESULTS

Apolipoprotein-induced cellular lipid release from HepG2 cells is shown in **Fig. 1**. There was a significant spontaneous release of cholesterol and choline-phospholipid by HepG2 cells (indicated at zero concentration of apolipoproteins). Both apoA-I and apoA-II, when exogenously added to the culture medium, induced further release of the lipids in a dose-dependent manner. The medium was analyzed for lipoprotein particles using gel-permeation HPLC (**Fig. 2**). Two major peaks were identified corresponding to LDL and HDL sizes by human plasma reference, whereas no significant peak was detected at the position of VLDL

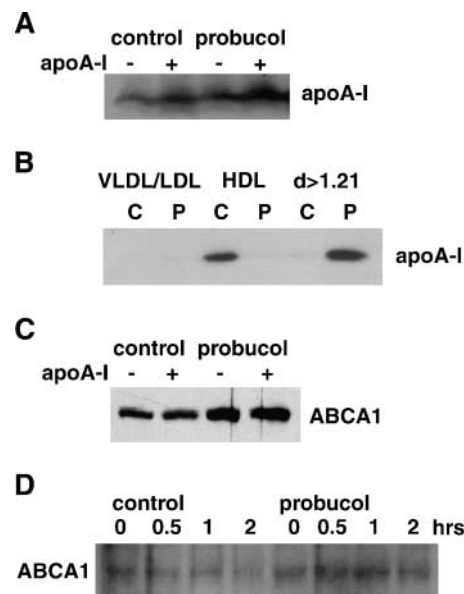


Fig. 7. Immunoblot analysis of apoA-I and ABCA1. A: ApoA-I in the conditioned medium of HepG2 cells was analyzed after preincubation with control LDL and probucol-containing LDL and subsequent incubation in the presence and absence of 10 μ g/ml apoA-I for 16 h. B: Density distribution of apoA-I in the medium. ApoA-I was analyzed in density subfractions of the conditioned medium of HepG2 cells preincubated with 50 μ g/ml control LDL (C) or probucol-containing LDL (P) for 24 h and then incubated for 16 h. The medium was fractionated by sequential ultracentrifugation for the VLDL/LDL fraction ($d < 1.063$ g/ml), the HDL fraction ($1.063 < d < 1.21$ g/ml), and the bottom fraction ($d > 1.21$ g/ml). Each fraction, corresponding to 10 μ l of the original medium, was dialyzed and analyzed for apoA-I. C: ABCA1 in the plasma membrane of HepG2 cells prepared as described for A. D: Clearance of ABCA1 in HepG2 cells. The cells were preloaded with control or probucol-containing LDL, and ABCA1 was examined after incubation with 71 μ M cycloheximide.

(**Fig. 2A**). The peaks were indeed verified to correspond to density classification of lipoprotein as fractions $d < 1.063$ and $d > 1.063$ (**Fig. 2C, E**). The conditioned medium incubated with 10 μ g/ml human apoA-I showed an increase of the HDL peak (**Fig. 2B, D, F**). Thus, increase of the release of cholesterol and phospholipid by apolipoprotein was shown to be attributable to the additional production of the HDL-size particles. Generation of lipoprotein was also examined for primary cultured mouse hepatocytes (**Fig. 3**). The cells were incubated with and without exogenous 10 μ g/ml human apoA-I for 24 h, and the conditioned medium was fractionated by ultracentrifugation. Cholesterol content in the HDL fraction ($d > 1.063$) was increased by 2-fold, and the VLDL-LDL fraction ($d < 1.063$) was insignificantly decreased. These results indicated that the system is functional both in HepG2 cells and in mouse hepatocytes for generation of the HDL-size lipoprotein by helical apolipoprotein.

To examine the contribution of ABCA1 to this system, the effect of an inactivator of ABCA1, probucol, was analyzed in those cells. Probucol was loaded to HepG2 cells, and the culture medium was analyzed by HPLC after 16 h of incubation with and without 10 μ g/ml apoA-I. The

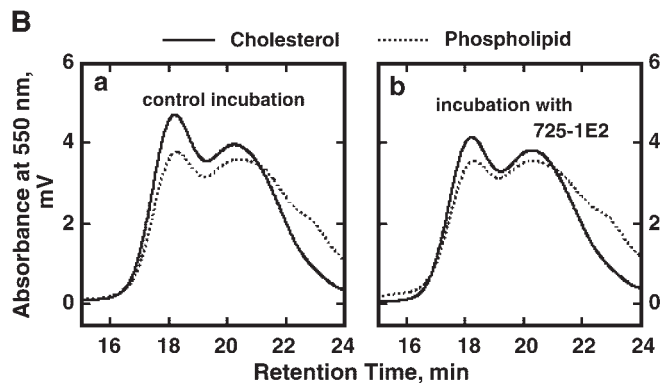
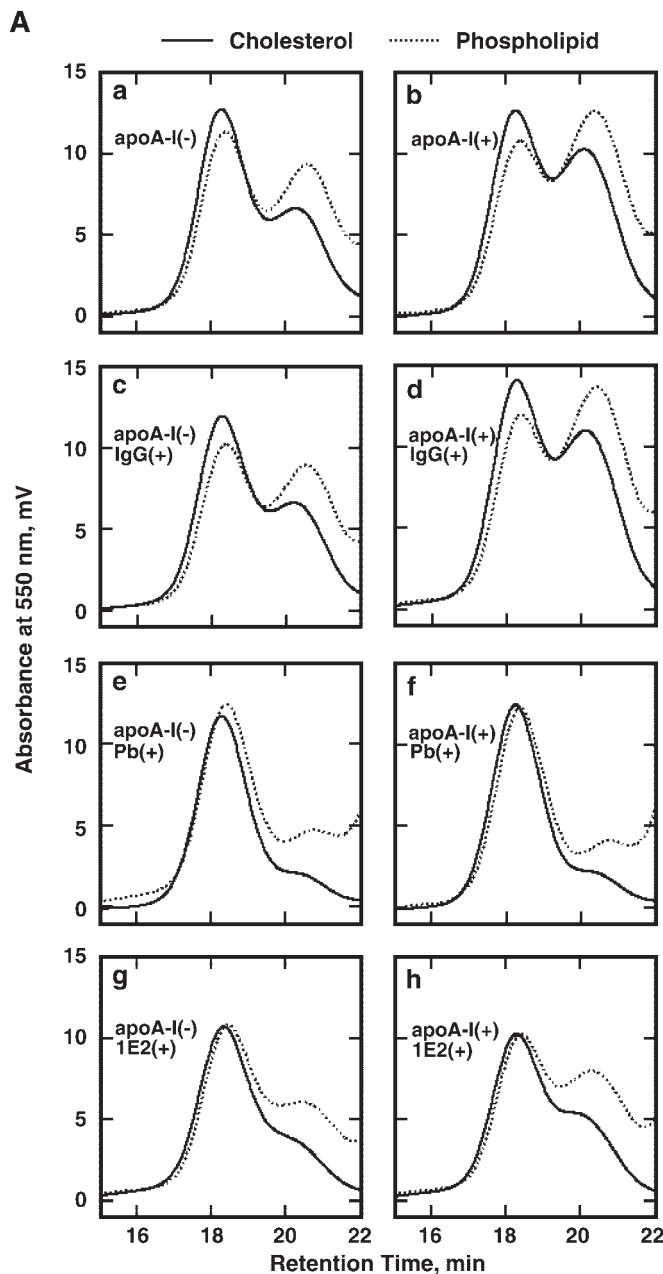


Fig. 8. Inhibition of HDL production by HepG2 cells by a monoclonal antibody against lipid-free apoA-I. **A:** HepG2 cells were incubated with 0.02% BSA-containing MEM- α medium in the presence of 1 mg/ml monoclonal antibody 725-1E2 specific to lipid-free apoA-I and 10 μ g/ml apoA-I for 16 h, and the medium was analyzed for its lipoprotein profile using HPLC with one gel-permeation column (Lipopropak XL; 7.8 mm \times 300 mm; Tosoh). **A-a:** The medium of the cells by plain incubation. **A-b:** The medium of the cells incubated in the presence of apoA-I. **A-c:** The medium after incubation in the presence of control mouse IgG. **A-d:** The medium incubated in the presence of apoA-I and control IgG. **A-e:** The medium of the probucol (Pb)-preloaded cells. **A-f:** The medium of the cell preloaded with probucol after incubation in the presence of apoA-I. **A-g:** The medium of the cells incubated in the presence of the free apoA-I-specific antibody 725-1E2. **A-h:** The medium of the cells incubated with the antibody 725-1E2 in the presence of apoA-I. **B:** The conditioned medium of HepG2 cells was incubated for 16 h in the absence (**B-a**) and presence (**B-b**) of the antibody, and the lipoprotein profile was analyzed by HPLC. Solid lines, cholesterol; dotted lines, choline-phospholipid.

data for cellular lipid and probucol are listed in **Table 1**. There was no difference in lipid composition between the control and probucol-loaded cells. The latter cells contained probucol at ~ 1 μ g/mg cell proteins, which was equivalent to our previous data with mouse peritoneal macrophages to which probucol was delivered by acetylated LDL (9). LDL- and HDL-size particles were identified in the medium, and the HDL fraction was increased by apoA-I (**Fig. 4A, B**). Probucol markedly decreased the HDL peak with both spontaneous production or exogenously added apoA-I (**Fig. 4C, D**). This finding was confirmed by electrophoretic analysis of the medium on an agarose gel (data not shown). The medium of mouse primary hepatocytes was also examined. The hepatocytes isolated from ABCA1-deficient mice produced no HDL, and apoA-I did not induce the production of HDL either (**Fig. 4E, F**). The effect of probu-

col was examined in hepatocytes prepared from C57Bl/6 mice by feeding control chow or 0.5% probucol-containing chow. The HDL peak decreased by probucol feeding (**Fig. 4G, H**). **Figure 5** shows the effect of probucol on the apoA-I-induced lipid release from HepG2 cells. The results indicated again that probucol inhibits the lipid release for both the baseline and an additional part induced by apoA-I. The cell-specific (reversible) binding of apoA-I was examined using 125 I-labeled apoA-I for HepG2 cells. Probucol inhibited the binding of apoA-I (**Fig. 5C**).

ApoB and apoA-I were analyzed in the HPLC fractions of the HepG2-conditioned medium (**Fig. 6**). ApoB was detected in the LDL fractions, and the probucol treatment did not alter this distribution. ApoA-I in the HDL fractions was markedly decreased by the probucol treatment. ApoA-I was also present in the free protein fractions, and this is

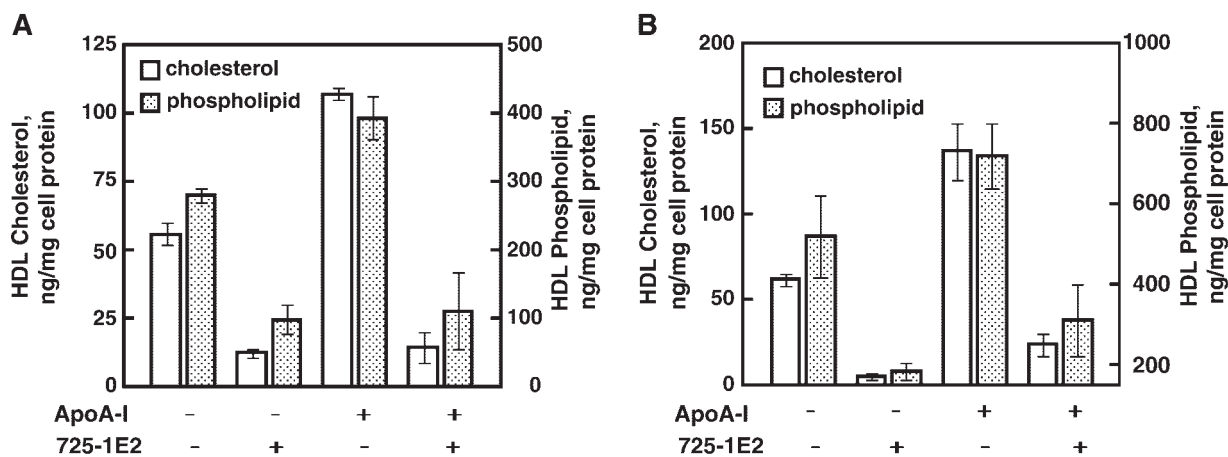


Fig. 9. Effect of free apoA-I-specific monoclonal antibody on HDL production by HepG2 cells. A: Cholesterol and phospholipid in HDL in the medium of HepG2 cells incubated in the presence of the antibody 725-1E2 and apoA-I, based on HPLC analysis. The data represent means \pm SEM of triplicate assays. B: Cholesterol and phospholipid in HDL in the medium of HepG2 cells incubated in the presence of the antibody 725-1E2 and apoA-I, based on ultracentrifugation analysis. Data represent means \pm SEM of triplicate assays.

consistent with the findings from the analysis of human plasma by HPLC in the diluted condition equivalent to the culture medium ($\times 250$) (data not shown). Although this HPLC method was thoroughly validated for lipoprotein lipid elution profile by its excellent correlation with the other gel-permeation method with a Superose column, apoA-I may dissociate from HDL particles during the analysis, presumably as a result of the use of 0.005% Brij-35 and extreme dilution of lipoprotein (17, 19).

Because of this limitation of the HPLC method, the medium was analyzed by ultracentrifugation to identify the status of apoA-I in the medium. When ABCA1 was inactivated by probucol in HepG2 cells, overall secretion of apoA-I was not influenced, as demonstrated in Fig. 7A. However, although apoA-I was localized in the HDL fraction ($1.063 < d < 1.21$ g/ml) in the control conditioned medium, it was found exclusively in the $d > 1.21$ g/ml fraction in the medium of probucol-loaded cells (Fig. 7B). Thus, apoA-I is secreted by the cells mostly in its free form when ABCA1 is inactivated.

Stabilization and increase of ABCA1 by additional apoA-I was not observed in this cell line, presumably because it is already affected by endogenously secreted apolipoproteins (Fig. 7C). However, ABCA1 was resistant against degradation in the probucol-loaded HepG2 cells (Fig. 7C, D) in the same manner as we demonstrated in fibroblasts (11).

Finally, we attempted to inactivate free apoA-I in the medium by trapping it using a monoclonal antibody to lipid-free apoA-I, 725-1E2 (15). As shown in Fig. 8, production of the HDL-size particles by both endogenous and exogenous apoA-I was effectively suppressed when this antibody was present in the medium during the incubation of HepG2 cells (Fig. 8Ag, Ah), whereas control IgG did not influence the profile (Fig. 8Ac, Ad). On the other hand, this antibody did not change lipoprotein profile when incubated with the HepG2-conditioned medium after removal of the cells (Fig. 8Ba, Bb). These results are quantitatively illustrated in Fig. 9A. Alternatively, the medium was analyzed by ultracentrifugation after the same experiments, and these

results are shown in Fig. 9B. Similar to the results of the HPLC analysis, HDL production was selectively suppressed by the lipid-free apoA-I monoclonal antibody 725-1E2.

DISCUSSION

The results of this work are summarized as follows. 1) HepG2 cells and mouse primary cultured hepatocytes produce HDL with endogenously synthesized apolipoprotein as well as with exogenously added apolipoprotein. 2) Production of HDL by both pathways is inhibited by the inactivation of ABCA1, but secretion of endogenous apoA-I was not decreased as much. ApoA-I is secreted as a free form when ABCA1 is inactivated. 3) A monoclonal antibody against lipid-free apoA-I, 725-1E2, suppresses the production of HDL by HepG2 cells but has no effect on lipoprotein when directly incubated with the HepG2-conditioned medium. Based on these observations, we conclude that the majority of HDL produced by hepatocytes are generated by the interaction of apoA-I that is secreted in a free or lipid-poor form with ABCA1 of the hepatocytes in an autocrine-like manner.

Many reports have characterized the HDL-like particles secreted in the culture medium by HepG2 cells and other liver cell lines (23–25). We essentially confirmed these findings with HepG2 cells and, in addition, demonstrated the increase of production of such particles by exogenously added apoA-I and apoA-II. Thus, the HepG2 cells have the same pathway to generate HDL as many other cells upon interaction with helical apolipoproteins, presumably dependent on ABCA1 (26).

Probucol has been shown to inhibit the apolipoprotein-cell interaction and accordingly to suppress the generation of HDL (9, 10). We recently demonstrated that this compound inactivates ABCA1 in the plasma membrane with respect to its activity as well as its calpain-mediated degradation (11). Probucol inhibited the production of

HDL, whether by endogenous or exogenous apolipoprotein, in hepatocytes. These findings suggested that endogenous and exogenous apolipoprotein, mainly apoA-I, generate HDL by a common mechanism, most likely by the ABCA1 pathway. Under this condition, the amount of the secreted apoA-I did not change, and it was secreted mostly in its free form (Fig. 7A, B). Therefore, the interaction of apoA-I with ABCA1 does not influence the rate of its secretion, but most apoA-I remains in a free form in the medium when it is unable to assemble HDL.

A monoclonal antibody raised against apoA-I, 725-1E2, has been characterized to be selective for lipid-free apoA-I (15). By using this antibody, we attempted to alter the production of HDL by HepG2 cells. In the presence of this antibody in the culture medium of HepG2 cells, the production of HDL markedly decreased. This finding strongly suggested that most apoA-I is secreted in its free form and trapped by the antibody, resulting in a decrease of HDL production. This view was supported by the finding that the incubation of the antibody with the HepG2-conditioned medium did not cause a change of the lipoprotein profile. Therefore, the effect of the antibody was not likely caused by the reaction with HDL after its generation by the cells.

It was reported that a certain portion of apoA-I (20%) was intracellularly lipidated in HepG2 cells and in primary cultured hepatocytes (8, 27), and some of the lipidation may not be mediated by ABCA1 (8). However, the present results indicated that the majority of HDL particles are produced by a common mechanism for endogenous and exogenous apolipoproteins, both of which can be inhibited by probucol. Also, most of the HDL production was inhibited by lipid-free apoA-I-specific antibody, indicating an autocrine mechanism: apoA-I is secreted as a free form and then interacts with hepatocytes to generate HDL. These findings by no means exclude the possibility of intracellular lipidation of apoA-I and the presence of the ABCA1-independent pathway as a minor source of HDL production.

ApoA-I is secreted as pro-apoA-I and converted to a mature form by a metalloprotease in blood plasma (28). Both pro-apoA-I and mature apoA-I were identically capable of generating HDL by reacting with ABCA1 (15), so perhaps there is no need to consider apoA-I maturation for this autocrine mechanism. **Fig.**

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