Heterogeneity of high density lipoprotein generated by ABCA1 and ABCA7

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Abstract The assembly of HDL by helical apolipoprotein and cellular lipid was studied using HEK293 cells to which ecdysone-inducible human ABCA1 or human ABCA7 was transfected. Expression of both ABCA1 and ABCA7 was induced linearly proportional to ponasterone A concentration in the medium. In the experimental conditions used, the ABC protein expression levels limited the rate of lipid release when the apolipoprotein concentration was high, and the apolipoprotein concentration was rate-limiting when the ABC protein expression levels were high. When ABCA1 expression increased in conditions in which it was rate-limiting, relative cholesterol content to phospholipid increased in the HDL produced. In contrast, it was constant when ABCA7 expression increased. To investigate the background mechanism, the HDL particles were analyzed by density gradient ultracentrifugation and high performance lipid chromatography. The ABCA1-mediated reaction produced two distinct HDLs, large cholesterol-rich and small cholesterol-poor particles, and the ABCA7-mediated reaction generated mostly small cholesterol-poor particles. The increase of HDL assembly with the increase of ABCA1 expression was predominant in large cholesterol-rich particles, whereas only small cholesterol-poor HDL increased as ABCA7 expression increased. We conclude that ABCA1 generates cholesterol-rich and cholesterol-poor HDL and that the former is more prominently dependent on the increase of ABCA1 expression. ABCA7 produces this HDL subfraction only as a very minor component.—Hayashi, M., S. Abe-Dohmae, M. Okazaki, K. Ueda, and S. Yokoyama. **Heterogeneity of high density lipoprotein generated by ABCA1 and ABCA7. *J. Lipid Res.* 2005.** 46: **1703–1711.**

Supplementary key words ATP binding cassette transporter A1 • ATP binding cassette transporter A7 • cholesterol • ecdysone-inducible

ABCA1 mediates the assembly of HDL with extracellular helical apolipoprotein and cellular lipid (1). This reaction is the major source of plasma HDL (2–4) and one of the rate-liming reactions for the regulation of its level (5, 6). The reaction mediates the generation of HDL particles with apolipoprotein, primarily recruiting cellular phospholipid (7). Cholesterol content in these particles is independently regulated by various cellular factors, potentially including protein kinase C and related signaling machineries (8–10), caveolin-1 (11), acyl-CoA:cholesterol acyltransferase (10, 12), and also perhaps other factors relating to intracellular cholesterol trafficking pathways (13). When ABCA1 is transfected to HEK293 cells, which otherwise do not express ABCA1, phospholipid and cholesterol are both released and cholesterol-rich HDL is generated upon incubation of the cells with apolipoprotein A-I (apoA-I) (14–16). ABCA7 also mediates the generation of HDL with apolipoproteins when transfected to HEK293 cells, but the relative cholesterol content to phospholipid in the HDL was lower than that produced by the ABCA1-mediated reaction (16, 17). The relative increase of cholesterol release seemed greater than that of phospholipid when ABCA1 protein level was upregulated by dibutyryl cAMP and phorbol ester (16), so we wondered whether the expression level of ABCA proteins is also a factor that regulates cholesterol content in the HDL. There are many reports that ABCA1 expression can be induced by various factors such as cAMP analogs (18–20), phorbol ester (10, 11), and ligands of liver X receptor or retinoid X receptor, including alteration of cellular cholesterol level (21–24). However, these compounds influence other cellular conditions and sterol metabolism and may not be suitable for examining the isolated effect of the expression level of the ABCA proteins. To address this question,

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Abbreviations: apoA-I, apolipoprotein A-I; DF, 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium; GFP, green fluorescent protein.

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we used the ecdysone-inducible mammalian expression system (Invitrogen), based on the ability of the insect molting hormone 20-H ecdysone to activate gene expression via the ecdysone receptor (25). Mammalian cells are not responsive to ecdysone or its analog ponasterone A, so the effect of these reagents is minimized other than the expression of a specific gene designed to react. With these cell systems, the expression of ABCA1 and ABCA7 was regulated quantitatively and the generation of HDL was examined accordingly.

Interestingly, the cholesterol content in HDL increased in a dose-dependent manner with ABCA1 but not with ABCA7. The ABCA1-mediated reaction produced large cholesterol-rich and small cholesterol-poor HDL particles, and the former predominantly increased as ABCA1 increased. On the other hand, ABCA7 produced predominantly small cholesterol-poor HDL even when its expression increased.

MATERIALS AND METHODS

Apolipoproteins

ApoA-I and apoA-II were isolated from fresh human HDL (26) and dissolved (19) in phosphate-buffered saline as stock solutions (1 mg/ml) to be stored at 4°C as described elsewhere.

DNA construction and transfection

Full-length cDNAs for human ABCA1 and human ABCA7 were cloned as described previously (15, 16) and subcloned into pIND vector (Invitrogen). ABCA1 cDNA within pEGFP-N1 was digested with *Not*I and ABCA7 cDNA within pEGFP-N1 was digested by *Not*I and *Hind*III. The purified inserts were ligated into pIND to obtain pIND-ABCA1-green fluorescent protein (GFP) and pIND-ABCA7-GFP. HEK293 expressing ecdysone receptor was purchased from Invitrogen and maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DF) supplemented with 10% (v/v) FBS (Hyclone) containing 400 μg/ml Zeocin (Invitrogen). The cloned cDNAs were transfected with Lipofectamine PLUS reagent (Invitrogen) according to the manufacturer's recommendation. Cells permanently introduced were selected with G418, and clones with higher level expression of the fusion proteins were further selected by FACStar (Becton Dickinson).

Evaluation of ABCA1 and ABCA7 expression levels

Total membrane fraction was prepared, and the expression of ABCA1 and ABCA7 was analyzed by immunoblotting using rabbit polyclonal anti-GFP antibody (Molecular Probes) (16).

Cellular lipid-release assay

Cells were seeded onto six-well trays at a density of 1.4×10^6 cells/well with 10% FBS-DF medium and incubated for 24 h. After treatment with ponasterone A of the ABCA1- and ABCA7-transfected cells for 17 and 24 h, respectively, cellular lipid release was induced in the presence of apoA-I or apoA-II (1–20 μg/ml) in DF containing 0.02% BSA and ponasterone A. Lipid was extracted from the medium, and free cholesterol and choline-phospholipid were measured in separate enzymatic assay systems (19).

Density gradient analysis

Cells were subcultured on 100 mm dishes at a density of 8.0×10^6 cells/dish, treated with ponasterone A, and stimulated by apoA-I (2 or 10 μg/ml) for 24 h in 5 ml/dish DF medium containing 0.02% BSA. The media from two dishes were combined and centrifuged to remove cell debris, and 8 ml of the supernatant was processed for sucrose density gradient ultracentrifugation (27). The solution was collected from the bottom of the tube

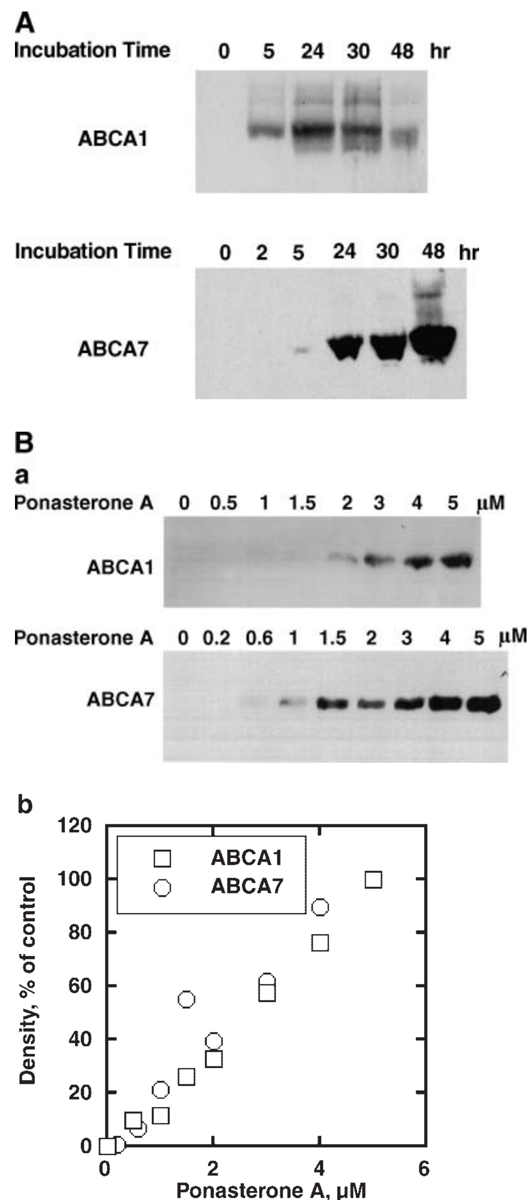

Fig. 1. Induction of green fluorescent protein (GFP)-tagged ABCA1 or ABCA7 expression by ponasterone A. A: The ecdysone-inducible cells were cultured for 24 h and incubated for the indicated times in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing 10% FBS in the presence of 5 μM ponasterone A. The membrane fraction (100 μg of protein) was analyzed for ABCA1 or ABCA7 by Western blotting against GFP. B: The ecdysone-inducible cells were cultured for 24 h and incubated for 17 h (ABCA1) or 24 h (ABCA7) in the presence of various concentrations of ponasterone A. The membrane fraction (100 μg of protein) was analyzed for ABCA1 or ABCA7 by Western blotting. The graph represents the density of the bands from the immunoblot quantitated by digital scanning in an Epson GT9500. The results are expressed as percentage of control (5 μM ponasterone A) for ABCA1 and ABCA7 (squares and circles, respectively).

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into 13 fractions. The cholesterol and choline-phospholipid contents as well as the density were determined for each fraction (19).

Lipoprotein analysis by HPLC

Lipoproteins in the conditioned medium were analyzed with an HPLC system (28) at Skylight Biotech, Inc. (Akita, Japan). The conditioned medium was centrifuged at 10,000 rpm for 5 min to remove cell debris, and a 200 μl aliquot was applied for an HPLC system using two tandem gel permeation columns (Lipopropak XL; 7.8 mm × 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 online enzymatic lipid detection system. 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 (29).

RESULTS

Induction of ABCA1 and ABCA7 by ponasterone A

GFP-tagged ABCA1 or ABCA7 cDNA with the ecdysone-inducible mammalian expression system was transfected to HEK293 cells that stably express the ecdysone receptor. It was previously confirmed that attachment of GFP to the C terminus of ABCA1 or ABCA7 did not influence their ability to produce HDL (16, 30). Expression of the ABCA proteins was examined by immunoblotting against GFP. With 5 μM ponasterone A treatment, expression of the GFP-tagged protein became apparent within 5 h, and it increased for 24 h in ABCA1 cells and for 48 h in ABCA7 cells (**Fig. 1A**). Therefore, 24 h induction was chosen as the optimum condition for ponasterone A to induce ABCA1 and ABCA7 for comparison. The dose-dependence of ABCA1 or ABCA7 expression was examined using various concentrations of ponasterone A. The immunoreacting bands of ABCA1-GFP and ABCA7-GFP both linearly increased ponasterone A in a dose-dependent manner (R^2 = 0.99 and 0.94, respectively) (Fig. 1B). At higher concentrations of ponasterone A (>10 μM), the expression levels seemed to reach a maximum (data not shown). The apoA-I-mediated lipid release increased by ponasterone A in a dose-dependent manner (**Fig. 2**). ABCA7 mediated cholesterol release less than ABCA1 with respect to its relative amount of phospholipid release.

Characterization of the lipid release mediated by ABCA proteins and apolipoproteins

Figure 3 demonstrates the release of cholesterol and phospholipid from HEK293 cells by apoA-I in the presence of ABCA1 and ABCA7. Cholesterol was almost all in a free form, and the amount of cholesteryl ester was negligible. When ABC protein expression is high (at high ponasterone A concentrations), apoA-I concentration limits the rate of lipid release. On the other hand, the expression levels of ABCA proteins limit the rate with the ponasterone A concentration used when apoA-I concentration is 10 and 20 μg/ml. **Figure 4** shows the results of similar experiments with apoA-II. The release of cholesterol and phospholipid by apoA-II appeared similar to the results

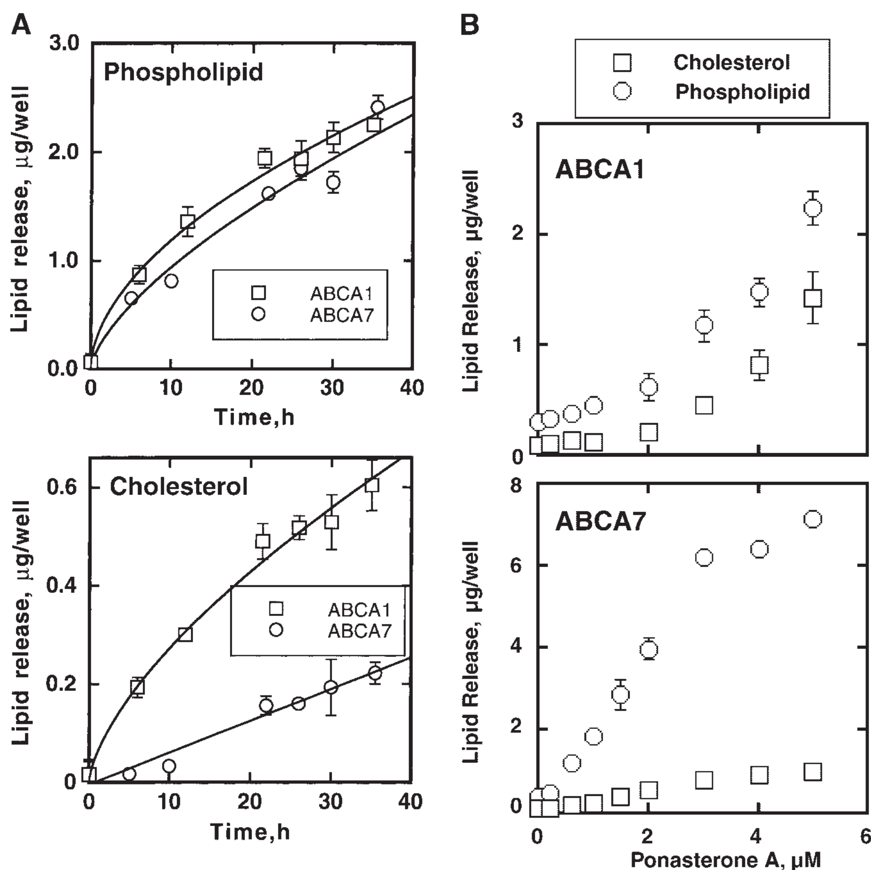

Fig. 2. Apolipoprotein A-I (apoA-I)-mediated release of cholesterol and choline-phospholipid. The ecdysone-inducible cells were incubated with the indicated concentrations of ponasterone A for 17 or 24 h. After washing with buffer H, the cells were incubated with apoA-I. A: Time-dependent profiles of the lipid release at 5 μM ponasterone A. B: Lipid release by apoA-I for 24 h at various concentrations of ponasterone A. Cholesterol (squares) and choline-phospholipid (circles) were determined in the medium. Data represent means ± SD for three samples.

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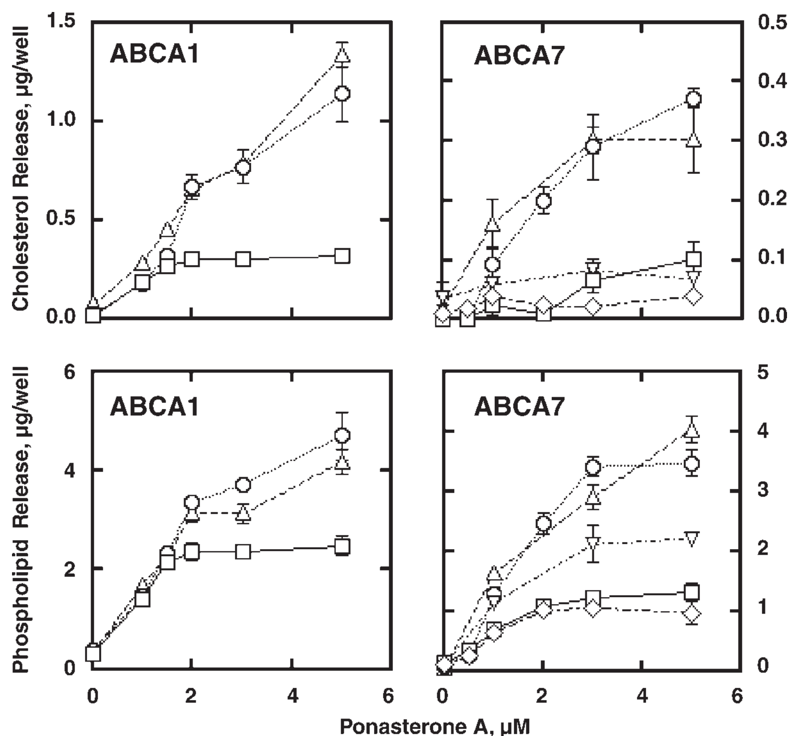

Fig. 3. Lipid release by apoA-I mediated with ABCA1 or ABCA7. The cells were induced for the expression of ABCA1 or ABCA7 in the presence of ponasterone A at 17 and 24 h, respectively. After washing, the cells were incubated with various concentrations of apoA-I [1, 2, 5, 10, and 20 µg/ml (diamonds, squares, inverted triangles, circles, and triangles, respectively)] for 24 h. Cholesterol and choline-phospholipid were measured in the medium. Total cellular cholesterol and choline-phospholipids were 17.5 ± 0.7 and 91.2 ± 2.5 µg/well in the ABCA1-expressing cells and 24.1 ± 2.5 and 116.2 ± 5.8 mg/well in the ABCA7-expressing cells (average ± SD for six samples).

with apoA-I with respect to rate-limiting profiles. When apoA-II concentration is as low as 1 and 2 µg/ml, it limits the rate of lipid release. On the other hand, ABCA1 or ABCA7 limits the rate when apoA-II concentration is as high as 5 and 10 µg/ml.

In the conditions in which ABCA proteins are rate-limiting, the ratio of cholesterol to phospholipid was examined in the released lipid (**Fig. 5**). When the expression of ABCA1 increases by increasing ponasterone A from 1 to 5 µM, the relative content of cholesterol in the released lipid increased by both apoA-I and apoA-II, from 0.1 to 0.4 and from 0.04 to 0.17, respectively (Fig. 5). In contrast, it remained constant at a low level when the expression of ABCA7 increased (Fig. 5).

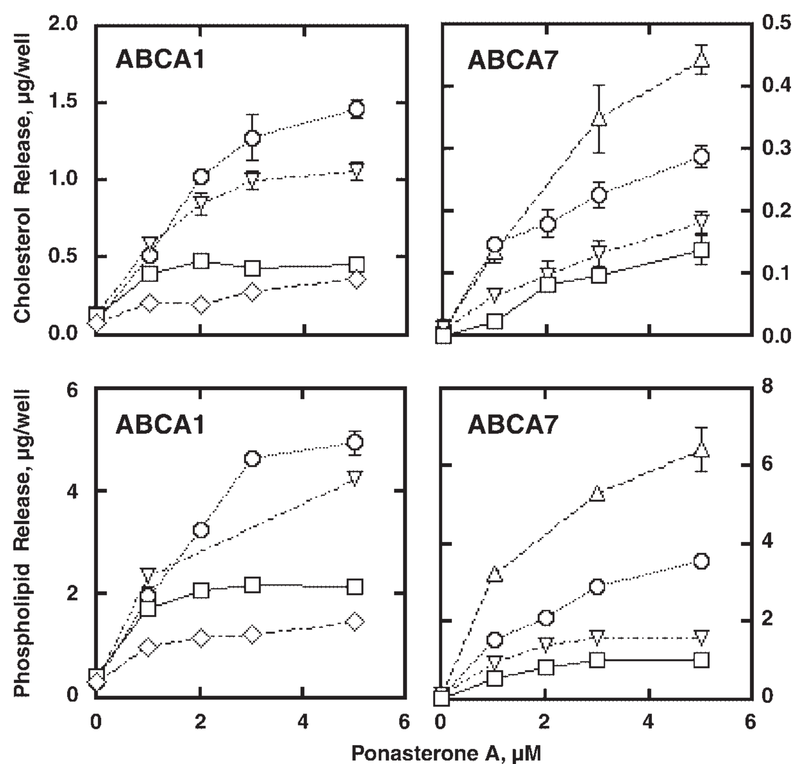

Fig. 4. Lipid release by apoA-II mediated with ABCA1 or ABCA7. The cells were induced for ABCA1 or ABCA7 in the presence of ponasterone A at 17 and 24 h, respectively. After washing, the cells were incubated with apoA-II [1, 2, 5, 10, and 20 µg/ml (diamonds, squares, inverted triangles, circles, and triangles, respectively)] for 24 h. Cholesterol and choline-phospholipid were determined in the medium.

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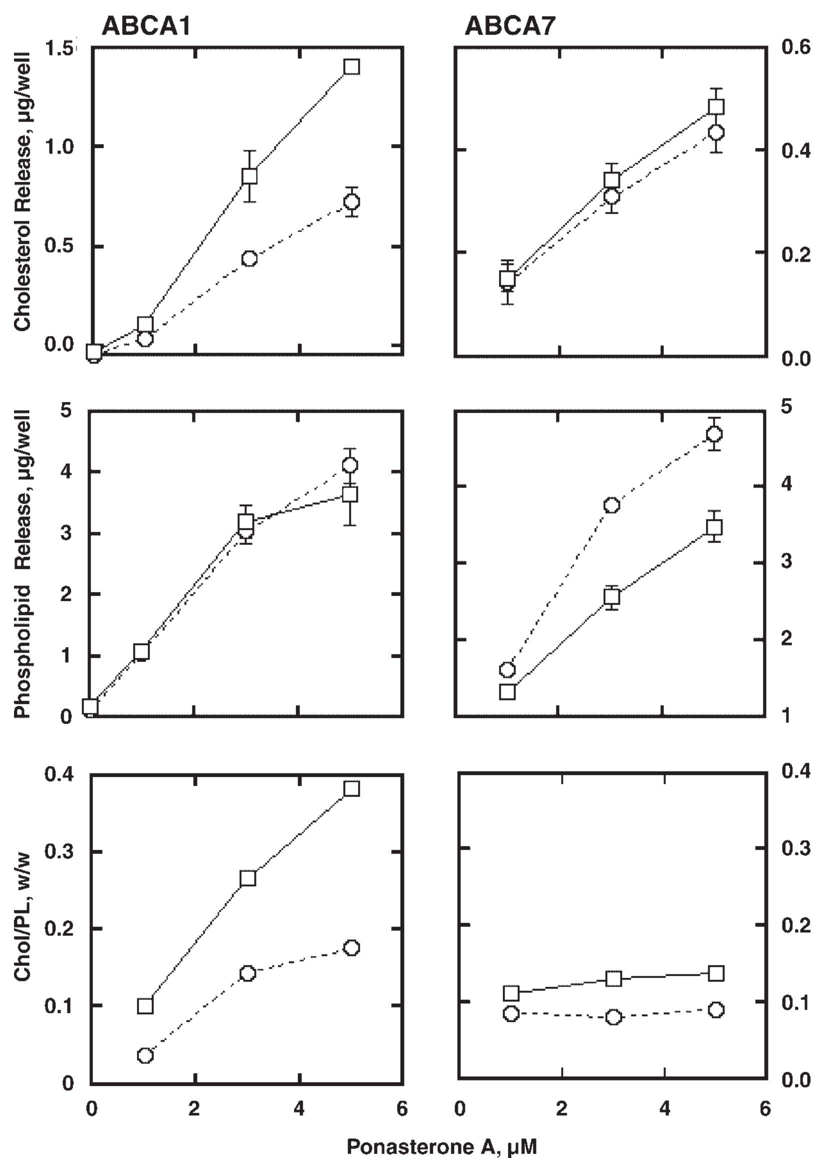

Fig. 5. Cholesterol-phospholipid ratio in the lipid released by apoA-I and apoA-II (squares and circles, respectively) mediated with ABCA1 or ABCA7. Expression of ABCA1 or ABCA7 was induced by ponasterone A, and cellular lipid release was induced by apolipoprotein (10 µg/ml). The ratio of cholesterol to phospholipid (Chol/PL) in mass is shown in the two bottom panels.

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Density gradient ultracentrifugation analysis of the conditioned media

The conditioned media with apoA-I of HEK293 cells transfected with ABCA1 or ABCA7 were analyzed by density gradient ultracentrifugation. Both cholesterol and phospholipid were recovered in the fractions, with a density range of HDL to confirm that the lipid was released to form HDL particles. The density of the peak was 1.08 g/ml with the ABCA1-transfected cells and 1.10–1.11 g/ml with the ABCA7-transfected cells (**Fig. 6**), consistent with our previous observation (16). Density profiles of lipid distribution for the ABCA1-transfected cells appear with a shoulder at ~1.11 g/ml, whereas those for the ABCA7-transfected cells were rather symmetric, with a slight irregularity in the lower density side.

Analysis of the media by HPLC

To examine the heterogeneity of the HDL generated, the conditioned media were analyzed by HPLC with a gel permeation column. Elution profiles of cholesterol and phospholipid were monitored online. **Figures 7** and **8** show the results with the apoA-I-conditioned media of the ABCA1- and ABCA7-transfected cells. The medium of the ABCA1-transfeted cells contained two peaks: large cholesterol-rich particles with a diameter of 13 nm, and small cholesterol-poor particles with a diameter of 10 nm. In contrast, the ABCA7-transfected cells generated a predominant peak that is small and cholesterol-poor, accompanied by large particles as a very minor component. These profiles were essentially the same in the conditioned media of HEK293 cells to which ABCA1 or ABCA7 was transiently expressed without the GFP tag (data not shown). When the lipid release was increased either by increasing ABCA1 expression (Fig. 7) or by increasing apoA-I (Fig. 8), a more prominent increase was induced in the large cholesterol-rich particles, and accordingly, the relative amount of cholesterol to phospholipid in the media increased.

In contrast, small cholesterol-poor particles were still major components of the HDL generated by the apoA-I-ABCA7-mediated reaction. This profile did not change even when ABCA7 expression was increased, resulting in

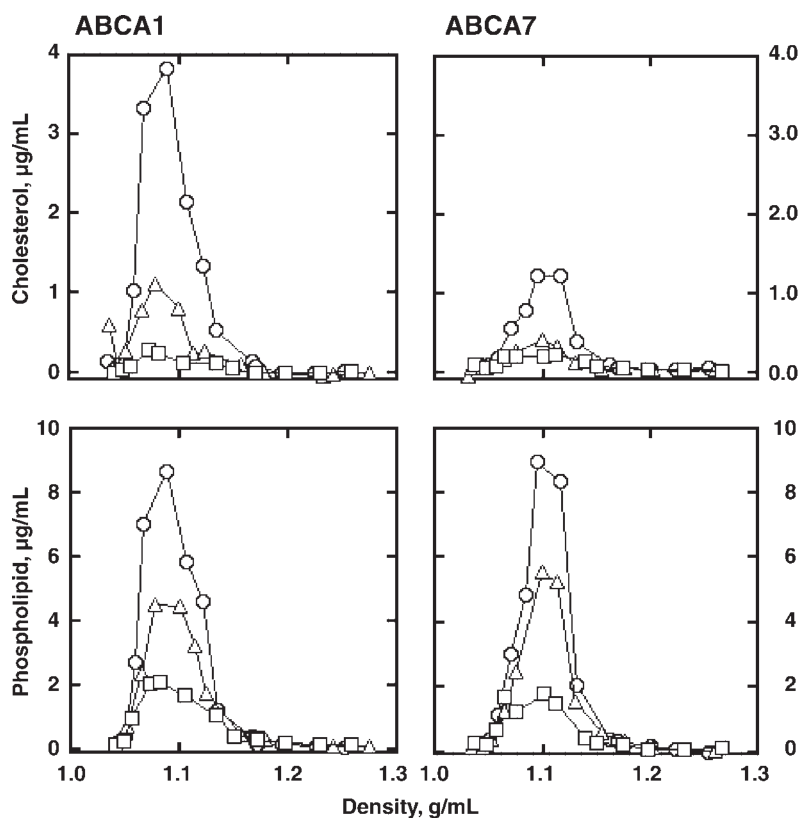

Fig. 6. Density gradient analysis of the lipids released by ABCA1- or ABCA7-expressing cells. The cells were induced for the expression of ABCA1 or ABCA7 by incubating with 5 mM ponasterone A for 17 and 24 h, respectively, and cellular lipid was released by apoA-I [2, 4, and 10 μg/ml (squares, triangles, and circles, respectively)] for 24 h. The medium was analyzed by density gradient ultracentrifugation as described in Materials and Methods. Each fraction was determined for density and concentration of cholesterol and phospholipid.

the relative cholesterol content remaining constantly low (Fig. 7). The same tendency was observed when apoA-I was rate-limiting and increased the generation of HDL from the ABCA7-transfeted cells (Fig. 8).

Component analysis of the peaks (31) estimated that the large HDL particles generated by the ABCA1-apoA-I reaction contained cholesterol with 35–38% weight of phospholipid and that the small HDL particles contained

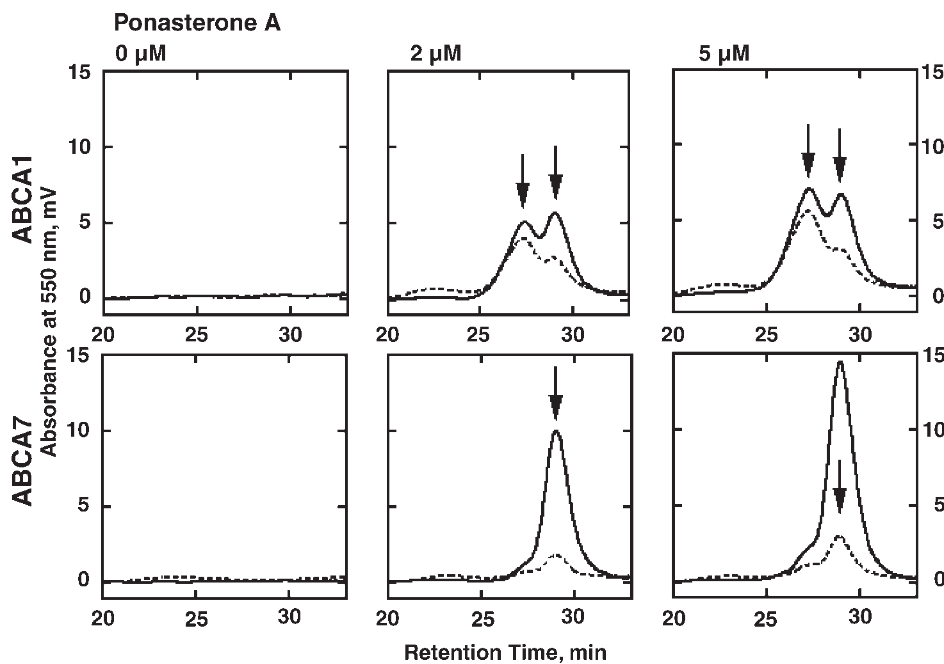

Fig. 7. HPLC analysis of the lipid released by ABCA1- or ABCA7-expressing cells. Lipoprotein analysis of the culture medium of ABCA1- or ABCA7-induced HEK293 cells by HPLC. The cells were induced for the expression of ABCA1 or ABCA7 by incubating with ponasterone A (2 and 5 μM) for 17 and 24 h, respectively, and cellular lipid was released by apoA-I (10 μg/ml) for 24 h. The conditioned medium (200 μl) was analyzed by the HPLC lipoprotein analysis system by monitoring cholesterol (broken lines) and phospholipid (solid lines). The arrows indicate eluting positions of the particles with diameters of 13 and 10 nm.

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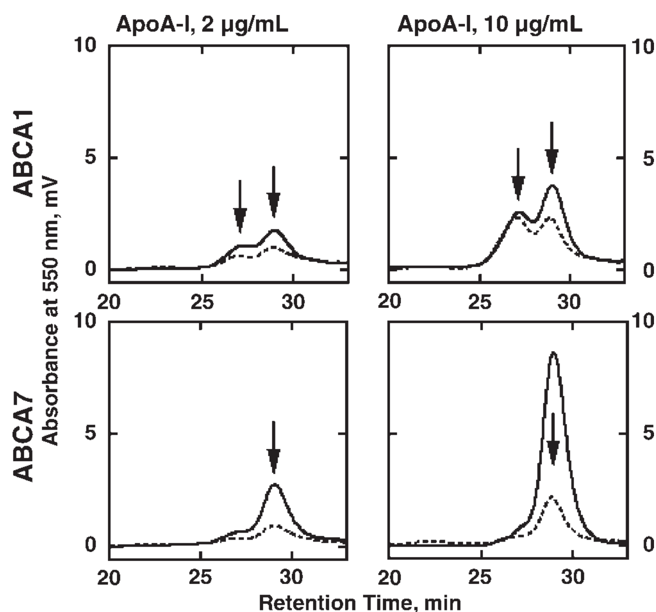

Fig. 8. HPLC analysis of the lipid released by ABCA1- or ABCA7-expressing cells. The cells were induced for the expression of ABCA1 or ABCA7 by incubating with ponasterone A (5 µM) for 17 and 24 h, respectively, and cellular lipid was released by apoA-I (2 and 10 µg/ml) for 24 h. The conditioned medium (100 µl) was analyzed by the HPLC lipoprotein analysis system by monitoring cholesterol (broken lines) and phospholipid (solid lines). The arrows indicate eluting positions of the particles with diameters of 13 and 10 nm.

10–13%. A major HDL component generated by the ABCA7-apoA-I reaction contained 8–11%.

DISCUSSION

ABCA1 is a key cellular factor for the generation of plasma HDL (2–6). ABCA7 was also demonstrated to mediate a similar reaction to ABCA1 to generate HDL in vitro (16, 17). There are several differences between the ABCA1- and ABCA7-mediated reactions with respect to cholesterol content in the HDL generated and posttranscriptional regulation. Recent studies indicated that ABCA7 does not play a major role in lipid release from macrophages (32, 33). However, it may contribute to a source of plasma HDL, at least in female mice (33), and it may be involved in lipid metabolism in kidneys (32) and adipose tissues (33).

The levels of expression of ABCA proteins may also affect these reactions, which could have significant influence on the nature of the products, such as their cholesterol content. However, the regulation of expression of ABCA proteins is multifactorial, including the liver X receptor/retinoid X receptor system (21–24), cAMP (18–20), the calcium-signaling pathway (34), and the peroxisome proliferator-activated receptor α-related system (35, 36). The content of cholesterol in the product HDL is regulated somewhat independently of the HDL assembly reaction itself, potentially with the involvement of such factors as caveolin-1 (11), protein kinase C-related signals (8–10), and intracellular cholesterol level and its esterification (10, 12). To characterize the HDL assembly reaction by ABCA proteins, it is thus important to control the expression level of these proteins without influencing other cellular factors.

We established the HEK293 cell systems with ecdysone-inducible ABCA1 and ABCA7. Both proteins are tagged with GFP, which was shown previously not to interfere with the function of either protein and enabled us to estimate the protein expression levels on a common immunoreactivity basis.

The results of the experiments are summarized below. Expression of ABCA1 and ABCA7 were both linearly proportional to the concentration of ponasterone A in the culture medium. The release of cellular cholesterol and phospholipid is limited by both ABCA protein expression levels and extracellular apolipoprotein levels. When ABCA proteins are rate-limiting, cholesterol release relative to phospholipid increased as ABCA1 expression increased, whereas it remained constant when ABCA7 expression increased. Increase of ABCA1 expression and apolipoprotein concentration both resulted in the increase of cholesterol content in HDL, and it was attributed to expansion of production of this component. On the other hand, ABCA7 mediated the generation of HDL particles that are predominantly small and cholesterol-poor. Thus, ABCA1 is more effective in releasing cellular cholesterol than is ABCA7 by producing large cholesterol-rich HDL. Both ABCA1 expression and apolipoprotein concentration increased relative to the release of cholesterol to phospholipid, and this was attributable to the expansion of production of this component.

Heterogeneity in nascent HDL particles produced by cell-apolipoprotein interaction has been reported in a previous study. When CHO-C19 cells were incubated with 8 µg/ml apoA-I for 24 h, 9.0 and 11.2 nm particles were produced (37). In another experiment with J774 macrophages, incubation with apoA-I led to the formation of more than one type of apoA-I-containing lipid particles, 9 and 12 nm in diameter, having cholesterol-phospholipid ratios of 1:1 to 1:3 (mol/mol), respectively (38). We also reported density profiles of HDL generated by peritoneal macrophages and apoA-I, which suggested similar heterogeneity of the HDL particles (8). The physicochemical and molecular bases for the size heterogeneity of discoidal HDL can be discussed (39), but it is not clear how such a mechanism can be applied when HDL is organized upon the apolipoprotein interaction with ABCA1 and membrane lipid. Lipid composition and particle size are related to the structure of specific domains of apoA-I on HDL (40). ABCA1 may induce specific modification of apoA-I conformation, either directly or indirectly, and such a change may cause parallel changes in its ability to bind to phospholipid bilayers and to the integration of cholesterol (41).

A higher expression level of ABCA1 causes the increase of large cholesterol-rich HDL particles. Although the exact mechanism by which ABCA1 functions in the membrane is not known, a few interesting findings have been

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reported. ABCA1 is said to alter the microenvironment of the plasma membrane and influence lipid-protein complex formation in the membrane (42). It forms an oligomeric structure and is related to its function for the biogenesis of nascent apoA-I-containing HDL (43). If oligomeric ABCA1 produces cholesterol-rich HDL, it is consistent with the view that a higher expression of ABCA1 and therefore an increase of oligomeric ABCA1 in the membrane may cause more production of cholesterol-rich particles. The apparent biphasic increase of the lipid release by the increase of ABCA1 expression (Fig. 2B) may also be consistent with this view. ABCA7 may not act in such a manner, and production of cholesterol-poor HDL simply increased when its expression increased. Tall and colleagues (32) suggested that the small amount of cholesterol release by the ABCA7-mediated reaction is attributable to its nonspecific acquisition by the phospholipid-HDL. It is not clear whether the small HDLs demonstrated in the present study gain cholesterol only by such a mechanism.

We have demonstrated a fundamental difference between ABCA1-mediated and ABCA7-mediated HDL assembly. ABCA1 mediates two types of HDL particles, large cholesterol-rich and small cholesterol-poor particles, and the generation of large cholesterol-rich particles is perhaps responsible for the efficient release of cell cholesterol. ABCA7 mediates the generation of only the latter type of particle. At higher expression, ABCA1 produces predominantly more large cholesterol-rich particles, whereas ABCA7 increases the production of only small cholesterol-poor HDL. The results presented here provide a new biochemical basis for understanding an HDL assembly pathway that involves ABCA1 or ABCA7.

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