Biogenesis of HDL by SAA is dependent on ABCA1 in the liver in vivo[S]

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Abstract **Serum amyloid A (SAA) was markedly increased in the plasma and in the liver upon acute inflammation induced by intraperitoneal injection of lipopolysaccharide (LPS) in mice, and SAA in the plasma was exclusively associated with HDL. In contrast, no HDL was present in the plasma and only a small amount of SAA was found in the VLDL/LDL fraction (d < 1.063 g/ml) after the induction of inflammation in ABCA1-knockout (KO) mice, although SAA increased in the liver. Primary hepatocytes isolated from LPS-treated wild-type (WT) and ABCA1-KO mice both secreted SAA into the medium. SAA secreted from WT hepatocytes was associated with HDL, whereas SAA from ABCA1-KO hepatocytes was recovered in the fraction that was >1.21 g/ml. The behavior of apolipoprotein A-I (apoA-I) was the same as that of SAA in HDL biogenesis by WT and ABCA1-KO mouse hepatocytes. Lipid-free SAA and apoA-I both stabilized ABCA1 and caused cellular lipid release in WT mouse-derived fibroblasts, but not in ABCA1-KO mouse-derived fibroblasts, in vitro when added exogenously. We conclude that both SAA and apoA-I generate HDL largely in hepatocytes only in the presence of ABCA1, likely being secreted in a lipid-free form to interact with cellular ABCA1. In the absence of ABCA1, nonlipidated SAA is seemingly removed rapidly from the extracellular space.**—Hu, W., S. Abe-Dohmae, M. Tsujita, N. Iwamoto, O. Ogikubo, T. Otsuka, Y. Kumon, and S. Yokoyama. **Biogenesis of HDL by SAA is dependent on ABCA1 in the liver in vivo.** ***J. Lipid Res.*** **2008.** 49: **386–393.**

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The acute phase response is characterized as various systemic metabolic changes caused by tissue injury or inflammation, including the induction of certain acute phase proteins and changes in lipid metabolism (1). Serum amyloid A (SAA) is a protein family that consists of acute phase and constitutive members (2), and acute phase SAA (SAA1 and SAA2 in human) is one of the major acute phase proteins. In the acute inflammatory response, SAA synthesis is induced in the liver by cytokines [interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α)], and its concentration in plasma increases up to 1,000-fold (2, 3). SAA in plasma is associated with HDL (4, 5), as the structure of SAA is very similar to that of amphiphilic helical apolipoproteins (6). The physicochemical properties of HDL are altered by acquiring SAA (5), but it is uncertain whether their biological functions are also differentiated in cholesterol transport or antiatherogenesis (1, 7, 8). It is also unclear whether the increase of SAA associated with HDL plays any role in the biological protection against acute or chronic inflammation.

ABCA1 is known to be essential for the biogenesis of HDL, as it mediates the interaction of amphiphilic helical apolipoproteins with cellular lipid to generate HDL particles and to remove cellular cholesterol (9–12). We demonstrated that SAA generates cholesterol-containing HDL directly from cellular lipid and that this reaction is mediated by ABCA1 and/or ABCA7 transfected to HEK293 cells (13).

In this paper, we extended our study on the mechanism for the biogenesis of SAA-HDL to the in vivo system. To focus on the role of ABCA1 in HDL biogenesis, we used ABCA1-knockout (KO) mice and investigated the generation of SAA-containing HDL in the acute phase response. We found that SAA biosynthesis and secretion were induced in the liver by acute inflammation regardless of the presence of ABCA1 but that HDL is not generated in the absence of ABCA1. Accordingly, no HDL increase was observed in ABCA1-KO mouse plasma in spite of the

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Abbreviations: apoA-I, apolipoprotein A-I; IL, interleukin; KO, knockout; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; SAA, serum amyloid A; SR-BI, scavenger receptor class B type I; TG, triacylglycerol; TNF, tumor necrosis factor; WT, wild-type.

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normal response of SAA production in the liver. These findings were consistent with those for apoA-I-mediated HDL biogenesis in liver.

MATERIALS AND METHODS

Reagents and antibodies

Lipopolysaccharide (LPS) prepared from *Escherichia coli* 0111: B4 was purchased from Sigma Chemical Co. (L 3024). ApoA-I was isolated from human plasma, stored at −80°C until use (14), and dissolved into a stock solution (1 mg/ml) for storage at 4°C (15). Recombinant human SAA corresponding to human SAA1a except for three amino acids was purchased from PeproTech EC (London, UK; catalog No. 300-13). A stock solution (1 mg/ml) was prepared according to the manufacturer's instructions and stored at 4°C as described previously (13). Concentrations of SAA and TNF-α were determined using ELISA kits (Biosource International Co.). Antibodies against mouse SAA (AF2948), mouse apoA-I (600-101-196), mouse ABCG1 (NB 400-132), and β-actin (A5316) were obtained from R&D Systems, Rockland Immunochemicals, Novus Biologicals, and Sigma, respectively. Monoclonal antibody to mouse and human ABCA1 (MABI98-7) was generated in rats against peptide CNFAKDQSDD-DHLKDLSLHKN, which is a common sequence of the C terminus of each protein, at the MAB Institute (Yokohama, Japan).

Animals

Heterozygotes of ABCA1-KO mice (ABCA1-hetero) (DBA/1-Abca1^{tm1Jdm}/J) (16) were purchased from Jackson Laboratory (Bar Harbor, ME). They were backcrossed onto C57BL/6 mice for more than eight generations, and the heterozygotes were intercrossed to obtain the offspring for experiments. The genotypes of the wild-type (WT), ABCA1-hetero, and ABCA1-KO mice were determined by PCR analysis of tail DNA, as described previously (17). Female mice at 8–16 weeks of age were used for experiments. The acute phase response was induced by intraperitoneal injection of 50 μg of LPS. The experimental procedure was approved by the Animal Welfare Committee of the Nagoya City University Graduate School of Medical Sciences according to institutional guidelines.

Cell culture

BALB/3T3 and CHO-K1 cells were obtained from the RIKEN cell bank and the American Type Culture Collection, respectively. Primary fibroblasts were prepared from the skin of 19–20 day old embryos. The skin tissue was cut into pieces of 1 mm^3 and placed in plastic dishes. After culturing for 10 days, the cells that migrated from the tissue pieces were collected with PBS containing 0.05% trypsin and 0.02% EDTA and stored at −150°C. Secondary cultured cells in the proliferating phase were subcultured and used for the experiments. Primary hepatocytes were prepared from mice as described previously (17). The cells were maintained in the medium supplemented with 10% (v/v) fetal calf serum (Gibco BRL) under a humidified atmosphere of 5% CO_2 and 95% air at 37°C. DMEM (high-glucose) was used for the primary hepatocytes, and a 1:1 mixture of DMEM and Ham's F12 medium (DF medium) was used for all other cells. The induction of SAA expression in hepatocytes was examined in two experimental protocols. For in vitro induction, hepatocytes were prepared from the mice with no treatment. After 3 h of incubation, the cells were washed with PBS and incubated with the medium containing 0.02% BSA and a cytokine mixture (IL-1β, IL-6, and TNF-α, 10 ng/ml each) for 16 h. For in vivo induction, mice were treated with 50 μg of LPS and the hepatocytes were prepared after 9 h. After 3 h of incubation, the cells were washed with PBS and incubated with the medium containing 0.02% BSA for 12 h. For lipid-release analysis, fibroblasts were subcultured in a six-well tray at a density of 5×10^5 cells/well and cultured with 10% fetal calf serum-DF medium. After 72 h, the medium was replaced and the cells were maintained for another 48 h. Then, the cells were washed with PBS and incubated in 1 ml/well DF medium containing 0.02% BSA and the compounds indicated. Lipid concentration in the medium was determined after 24 h for cholesterol and choline-phospholipids by specific enzymatic assays as described previously (15).

Lipoprotein analysis

The plasma VLDL/LDL, HDL, and protein fractions were isolated by the ultracentrifugal flotation procedure, and cholesterol content in each fraction was measured using a colorimetric enzyme assay kit (Kyowa Medex Co.) (17). An HPLC system with two tandem gel permeation columns was used to evaluate the size distribution of plasma lipoprotein particles (18, 19) (Skylight Biotech, Inc., Akita, Japan). Samples were diluted 20 times and analyzed at a flow rate of 350 μl/min by monitoring the concentrations of choline-phospholipid, total cholesterol, and triacylglycerol (TG), with absorbance at 585 nm for choline-phospholipid and at 550 nm for total cholesterol and TG.

RT-PCR

Total RNA was prepared and reverse-transcribed by SuperScript III (Invitrogen) with random oligonucleotide primers. Primers used for quantitative RT-PCR were as follows: for SAA, 5′-AGA TGC TCT CTG GGG AAA CA-3′ (forward) and 5′-TAC CCT CTC CTC CTC AAG CA-3′ (reverse); for ABCG1, 5′-TCC ATC GTC TGT ACC ATC CA-3′ (forward) and 5′-TTC AGA CCC AGA TCC CTC AG-3′ (reverse); for apoA-I, 5′-ACG TAT GGC AGC AAG ATG AAC-3′ (forward) and 5′-AGA GCT CCA CAT CCT CTT TCC-3′ (reverse). Primers for 18S rRNA and ABCA1 were prepared as described previously (20). Results were normalized to 18S rRNA.

Statistical analysis

Data were analyzed by one-way ANOVA. $P < 0.05$ by Scheffé's test was accepted as statistically significant.

RESULTS

Size-exclusion HPLC analysis demonstrated the absence of plasma HDL and very small amounts of other lipoproteins in ABCA1-KO mice (**Fig. 1**), reflecting very low plasma cholesterol levels in ABCA1-KO mice and ~50% in WT mice and ABCA1-hetero mice (see supplementary data 1), consistent with a previous report (16). Acute inflammation was induced by peritoneal injection of LPS (50 μg/mouse) in WT, ABCA1-hetero, and ABCA1-KO mice. LPS injection caused increases in plasma HDL-cholesterol and HDL-phospholipid in WT and ABCA1-hetero mice after 24 h but not in ABCA1-KO mice (Fig. 1, **Table 1**). With the LPS treatment of WT mice, the HDL increase accompanied shifting of its eluting position earlier (Fig. 1A, C). In ABCA1-KO mice, there was no HDL peak even after the LPS injection (Fig. 1B, D). Monitoring TG concentration revealed that LPS injection caused decreases of VLDL and

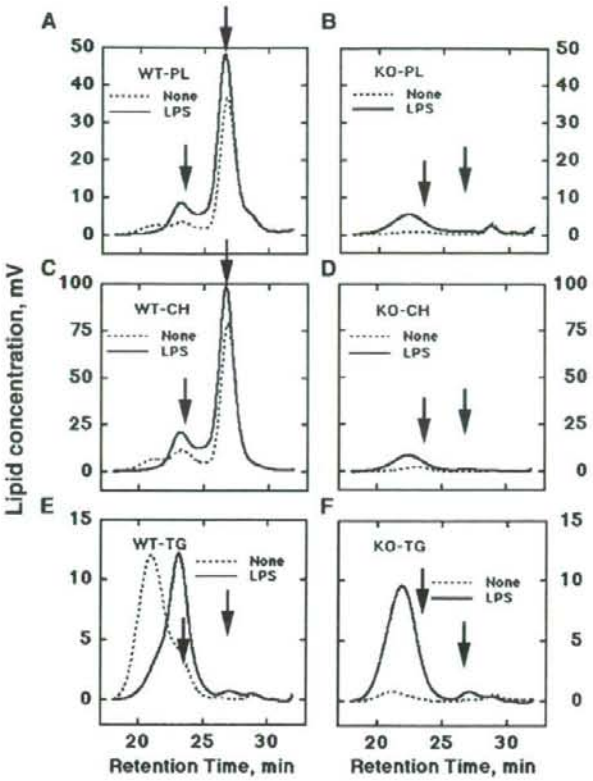

Fig. 1. Effect of lipopolysaccharide (LPS) treatment on mouse plasma lipoprotein. Wild-type (WT) and ABCA1-knockout (KO) mice were treated with (solid lines) and without (dashed lines) LPS. Plasma was collected at 24 h after the treatment and analyzed by molecular sieve HPLC. A and B represent monitoring of choline-phospholipid (PL), C and D represent monitoring of total cholesterol (CH), and E and F represent monitoring of triacylglycerol (TG). Standard eluting positions for peaks of human plasma LDL and HDL are at 23.4 and 26.7 min, respectively, as indicated by arrows. Lipid concentration was monitored as described in the text, being expressed in mV and calibrated as 55.6 nM/mV for choline-phospholipid and 30.0 nM/mV for cholesterol and TG.

increases of TG-rich LDL in both WT mice (Fig. 1E) and ABCA1-KO mice (Fig. 1F). Agarose gel electrophoresis revealed that the electrophoretic mobility of HDL in WT mice became slower and faint lipid staining appeared in a slow HDL fraction in ABCA1-KO mice after LPS injection (see supplementary data I).

Table 1 shows changes in HDL-cholesterol, SAA, and TNF-α in mouse plasma after LPS injection. Although HDL cholesterol increased markedly in WT and ABCA1-hetero mice, it did not increase in ABCA-KO mice. No SAA was detected in plasma without LPS injection. SAA was increased significantly in WT and ABCA1-hetero mice, whereas it remained at a very low level in the ABCA1-KO mouse plasma. SAA concentration in ABCA1-hetero mouse plasma was about half of that in WT mice. Because LPS is known to induce TNF-α, a mediator and one of the major stimulants for SAA production (2), TNF-α concentration in plasma was measured to determine whether ABCA1-KO mice respond to LPS. Transient increases of plasma TNF-α were detected at 2 h after the LPS injection in all WT, ABCA1-hetero, and ABCA1-KO mice, and these was diminished at 24 h. The increase was highest in ABCA1-KO mice.

As shown in **Fig. 2A**, SAA that appeared in plasma was associated with HDL in WT mice, whereas SAA was barely found in ABCA1-KO mouse plasma and was associated with the VLDL/LDL fraction. In contrast, substantial induction of SAA was identified in the liver of ABCA1-KO mice, essentially as much as in WT mice (Fig. 2B). To examine whether SAA is secreted from the liver, we prepared primary hepatocytes from WT and ABCA1-KO mice. Our attempt at in vitro induction of SAA by the cytokine mixture was unsuccessful, probably because of their cytotoxicity in our experimental conditions of using serum-free medium (data not shown), inconsistent with a previous report (21). Therefore, in vivo induction was used as pretreatment of mice with LPS. The message of SAA in the liver was increased markedly in this condition, as discussed below. SAA was detected in the conditioned medium of the hepatocytes prepared from untreated WT mice, and the LPS pretreatment increased it. SAA in the medium of WT hepatocytes was all recovered in the HDL fraction. In contrast, all of the SAA induced by LPS was found in the protein fraction defined as $d > 1.21$ g/ml in the medium of ABCA1-KO mouse hepatocytes (Fig. 2C). ApoA-I was found in the conditioned medium in every experimental condition. Secretion of apoA-I decreased by LPS in both genotypes. The distribution of apoA-I between HDL and free protein fractions was similar to that of SAA

TABLE 1. Effects of LPS treatment on mice

Variable	Time after Injection of LPS	Genotype		
		WT	Hetero	KO
HDL-cholesterol (mg/dl)	0 h	45.4 ± 5.3	32.5 ± 4.2	0.5 ± 0.2
	24 h	81.0 ± 7.0[a]	57.8 ± 11.6[a]	1.1 ± 1.5
SAA (mg/ml)	0 h	0.25 ± 0.26	0.01 ± 0.00	0.01 ± 0.02
	24 h	14.20 ± 1.47[a]	8.20 ± 4.20[a]	1.24 ± 1.33[b,c]
TNF-α (ng/ml)	0 h	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01
	2 h	0.42 ± 0.15[c]	0.55 ± 0.31[a]	1.48 ± 0.33[a,b,c]
	24 h	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01

Hetero, heterozygotes of ABCA1-KO mice; KO, knockout; LPS, lipopolysaccharide; SAA, serum amyloid A; TNF, tumor necrosis factor; WT, wild-type. Plasma was collected before and 24 h after LPS treatment. HDL-cholesterol SAA and TNF-α were measured as described in the text. Each value represents the mean ± SD (n = 8 for HDL-cholesterol, n = 6–8 for SAA, and n = 3 for KO at 24 h, 6 for KO at 0 and 2 h, 8 for WT and Hetero at 0 h, and 10 for WT and Hetero at 2 and 24 h for TNF-α).

[a] Significant statistical difference ($P < 0.001$) from the 0 h group with matched genotype.
[b] Significant statistical difference ($P < 0.001$) from the WT group.
[c] Significant statistical difference ($P < 0.001$) from the Hetero group.
[d] Significant statistical difference ($P < 0.01$) from the WT group.
[e] Significant statistical difference ($P < 0.01$) from the Hetero group.

(Fig. 2C). To determine whether the generation of SAA-containing HDL depends on ABCA1, we examined SAA-mediated lipid release from skin fibroblasts (**Table 2**). Both apoA-I and SAA induced the release of cholesterol and phospholipid from WT mouse fibroblasts. However, no lipid release was observed from ABCA1-KO mouse fibroblasts. The data indicated that SAA and apoA-I are both secreted by hepatocytes regardless of ABCA1 genotype, although the production of HDL with these proteins requires ABCA1.

Figure 3 demonstrates changes in various messages in the liver after LPS injection to the mice. In WT mice, the increase of SAA mRNA was apparent as early as 2 h after the injection, continued to increase for 16 h, and returned to the basal level at 48 h. ApoA-I mRNA was decreased by LPS injection. ABCA1 mRNA increased soon after the LPS injection, reached a peak at 2 h, and decreased to the control level at 24 h. ABCG1 mRNA was not affected during the experimental time course. The changes in mRNA

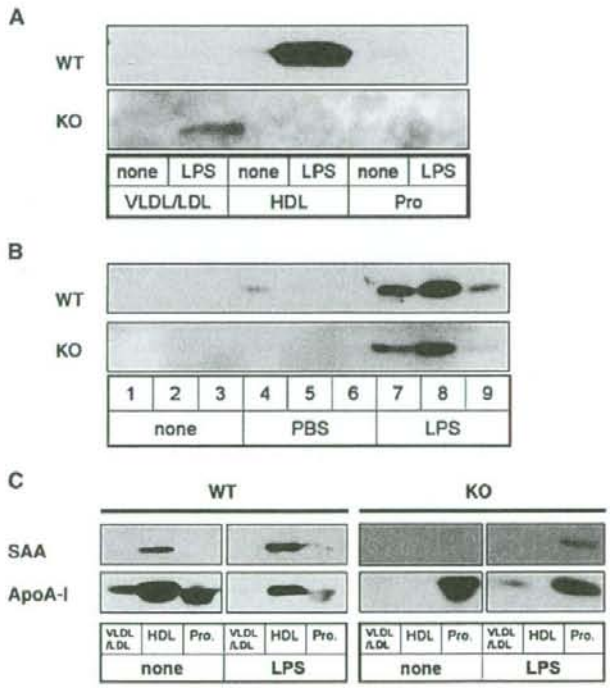

Fig. 2. Association of serum amyloid A (SAA) with lipoprotein fractions. A: Plasma SAA distribution in mice after LPS injection. Plasma was collected at 0 h (none) and 24 h (LPS) after LPS injection from WT and ABCA1-KO mice, and SAA was analyzed by Western blot analysis in VLDL/LDL, HDL, and protein (Pro) fractions. Lipoprotein fractions were equivalent to 50 μl of plasma, and protein fractions were equivalent to 5 μl. B: SAA production in the liver after LPS treatment of mice. Liver SAA protein was analyzed before (none) and after the injection of PBS or LPS in WT and ABCA1-KO mice. Twenty-four hours after the injection, mice were perfused with PBS to wash out plasma SAA and the liver was collected for Western blot analysis (100 μg protein/lane). C: Analysis of the culture medium of the primary hepatocytes isolated from WT and ABCA1-KO mice for SAA and apolipoprotein A-I (apoA-I). WT and ABCA1-KO mice were treated with LPS or untreated (none), and the liver was removed after 6 h. Hepatocytes were prepared and placed onto 60 mm dishes at a concentration of 1.8×10^6 cells/dish. After 3 h of incubation, the cells were washed with PBS and incubated with medium containing 0.02% BSA. After another 12 h of incubation, the conditioned medium was collected. SAA and apoA-I in the medium of VLDL/LDL, HDL, and protein (Pro) fractions were analyzed by Western blotting.

TABLE 2. Cellular lipid release mediated by exogenous lipid-free apoA-I and SAA

Lipid Release	Genotype	Treatment		
		None	ApoA-I	SAA
Cholesterol	WT	0.21 ± 0.03	1.29 ± 0.14*	1.22 ± 0.05*
	KO	0.10 ± 0.02	0.10 ± 0.03	0.25 ± 0.05
Phospholipid	WT	1.07 ± 0.05	2.78 ± 0.40*	2.10 ± 0.02*
	KO	0.69 ± 0.13	0.79 ± 0.11	0.80 ± 0.03

Fibroblasts prepared from WT mice and ABCA1-KO mice were incubated with 0.02% BSA/DF medium (see Materials and Methods) containing 10 μg/ml apoA-I, 10 μg/ml SAA, or 0.02% BSA/DF medium alone (none) for 24 h. Cholesterol and phospholipid in the medium were measured as described in Materials and Methods. Each value represents μg/well (mean ± SD; n = 3).

*Significant statistical difference ($P < 0.001$) from the none group with a matched genotype.

levels of SAA, apoA-I, and ABCG1 in ABCA1-KO mice were similar to those observed with WT mice. ABCA1 protein, however, increased and reached a peak at 16 h after LPS injection and remained higher than the basal level even at 48 h (**Fig. 4A**). In contrast, ABCG1 protein was unaffected by LPS injection (Fig. 4A).

As the time courses of the increase of ABCA1 and SAA mRNA were different, SAA protein is unlikely to induce an increase of ABCA1 mRNA. On the other hand, helical apolipoproteins such as apoA-I stabilize ABCA1 protein against its calpain-mediated proteolysis (22). Therefore, we investigated the effect of SAA on the degradation of ABCA1 protein in vitro. As shown in Fig. 4B, the clearance of ABCA1 protein in primary cultured fibroblasts and BALB/3T3 cells was retarded by SAA and apoA-I. Similar results were demonstrated with CHO-K1 cells (data not shown). Therefore, prolonged increase of ABCA1 protein in the liver after LPS treatment was likely attributable to its stabilization by SAA protein.

ABCA1 protein level was also examined in extrahepatic organs. As shown in Fig. 4C, liver ABCA1 protein level was higher in LPS-treated animals than in the untreated control group even at 24 h after injection, when the mRNA level had already returned to the original level, as indicated in Fig. 3. ABCA1 protein levels in the brain and adrenal gland were not affected significantly by LPS injection.

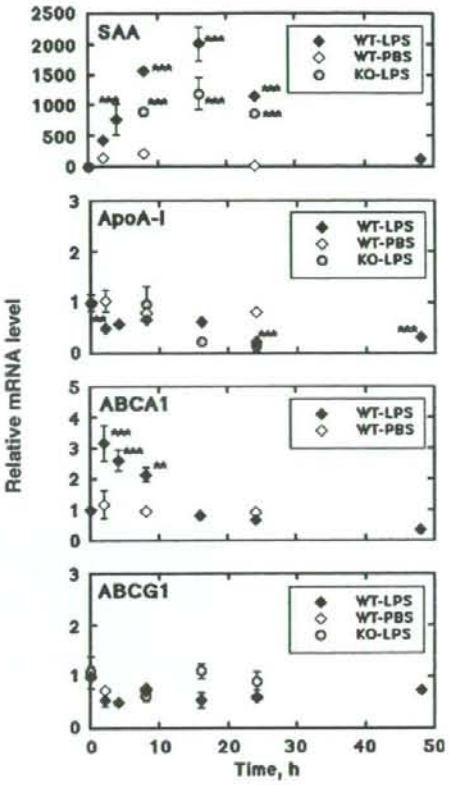

Fig. 3. Changes in the expression of SAA, apoA-I, ABCA1, and ABCG1 in the liver of ABCA1-KO (circles) and WT (diamonds) mice by LPS injection. Mice were treated with LPS (or with PBS), and the liver was collected at the times indicated (0, 2, 4, 8, 16, 24, and 48 h for WT mice and 0, 4, 8, 16, and 24 h for ABCA1-KO mice). Levels of SAA, apoA-I, ABCA1, and ABCG1 mRNA were determined by quantitative RT-PCR. Data represent means ± SD (n = 3) relative to the level of WT mice at 0 h. Significant statistical differences from the 0 h data are indicated with asterisks: ** $P < 0.01$, *** $P < 0.001$.

DISCUSSION

We investigated the mechanism for the biogenesis of SAA-HDL and a role of ABCA1 in a mouse model using ABCA1-KO mice. The results showed that the production and secretion of SAA in the liver were induced in an acute phase response to inflammation preceded by an increase of TNF-α in plasma. Plasma SAA was increased markedly at this stage, being associated with HDL only when ABCA1 was present. Expression of the ABCA1 gene was enhanced in the acute phase, likely independent of SAA induction, and degradation of the ABCA1 protein was presumably retarded in the liver because of its stabilization by SAA.

The involvement of scavenger receptor class B type I (SR-BI) has been suggested in the biogenesis of SAA-HDL (23). However, our previous data demonstrated that SAA-HDL biogenesis was SR-BI-independent (13). Although the expression of SR-BI mRNA in the liver was the same between WT and ABCA1-KO mice (Hu et al., unpublished data), the hepatocytes of ABCA1-KO mice did not produce SAA-HDL (Fig. 2C). The reports that the SAA-mediated cholesterol release from ABCA1-expressing cells was enhanced by SR-BI (24) and that SR-BI accelerates cellular lipid release only to the "lipidated" SAA in the absence of ABCA1 (25) indicate that the initial biogenesis of SAA-HDL particles depends on ABCA1 and that SR-BI may further enhance lipid release to the HDL. ABCA7 also mediated HDL biogenesis when transfected

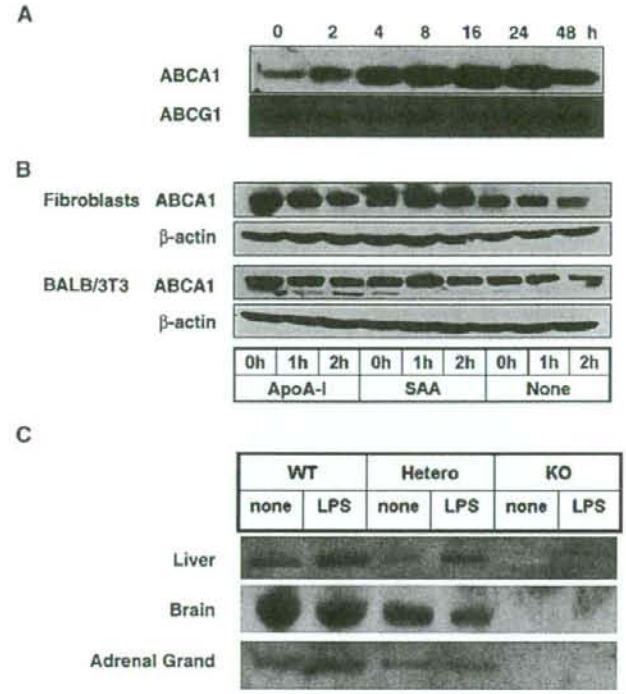

Fig. 4. A: Protein levels of liver ABCA1 and ABCG1 in LPS-treated WT mice at the indicated times. Each protein was analyzed by Western blotting (100 μg protein/lane). B: Retardation of the ABCA1 decay rate by lipid-free SAA and apoA-I. Fibroblasts prepared from WT mice and BALB/3T3 cells were incubated with 0.02% BSA/DF medium (see Materials and Methods) containing 20 ng/ml cycloheximide only (none), 20 ng/ml cycloheximide and 10 μg/ml apoA-I (apoA-I), or 20 ng/ml cycloheximide and 10 μg/ml SAA (SAA) for 0, 1, and 2 h. Cells were collected and ABCA1 was analyzed by Western blotting (100 μg protein/lane). β-Actin was analyzed as a control. C: ABCA1 protein levels after LPS treatment. WT, ABCA1-hetero (heterozygotes of ABCA1-KO mice), and ABCA1-KO mice were treated with LPS for 24 h. ABCA1 was analyzed in the liver, brain, and adrenal glands by Western blotting (100 μg protein/lane).

and overexpressed (13). However, endogenously expressed ABCA7 is not expressed on the cell surface and may not be involved in HDL biogenesis (20, 26). This view supports the finding that SAA does not produce HDL with the cells of ABCA1-KO mice, in which ABCA7 expression is increased (20). Therefore, there is unlikely to be an alternative pathway(s) for SAA-HDL production to the ABCA1-dependent mechanism, at least in vivo.

We demonstrated using an antibody specific to lipid-free apoA-I that HDL is generated by apoA-I in an autocrine manner in hepatocytes (17). The present findings for SAA-mediated HDL biogenesis in the mouse liver seem similar to those for apoA-I and consistent with an autocrine mechanism. However, we do not exclude an alternative interpretation: that HDL is preformed by helical apolipoproteins and SAA displaces those from HDL afterward (5), although apoA-I is not required for the production of SAA-HDL (27). SAA can be secreted partially as a free form by hepatocytes even in the presence of ABCA1 (Fig. 2C) (28), perhaps indicating that the ABCA1 expression level is rate-limiting for HDL biogenesis when SAA is overproduced. The lack of HDL production by LPS injection in apoA-I/apoE-deficient mice (29) may reserve the possibility that the synthesis of apoE-HDL is a prerequisite for SAA-HDL formation. Like lipid-free apoA-I, lipid-free SAA seems to be removed rapidly from the extracellular space by an unknown mechanism in vivo, as it does not accumulate in plasma.

The time-dependent increase of SAA mRNA was similar between ABCA1-KO and WT mice (Fig. 3), as was the change in plasma TNF-α concentration (Table 1), indicating that there is no fundamental difference in acute phase response reactions in ABCA1-KO mice. The synthesis and secretion of SAA by ABCA1-KO hepatocytes may be somewhat less than in WT hepatocytes (Fig. 2A, B). When the production of SAA was adjusted between the ABCA1-KO and WT mice using reduced doses of LPS (12.5 and 25 μg) to the WT mice (see supplementary data IIA), plasma cholesterol increased and all SAA was recovered in HDL (see supplementary data IIB, IIC). Thus, low SAA-HDL production in ABCA1-KO mice is not attributed to the relatively low SAA production. Although SAA is associated mainly with HDL in the acute phase, it may also associate with other lipoproteins (30, 31). The finding of trace amounts of SAA associated with the VLDL/LDL fraction in ABCA1-KO mice (Fig. 2A) should be consistent with those previous observations.

LPS injection caused a rapid but transient increase of ABCA1 mRNA in the liver. It reached a maximum at 2 h and returned to the original level at 16 h in our experimental conditions (Fig. 3). As ABCG1 mRNA was not affected (Fig. 3), a common positive transcription factor for the ABCA1 and ABCG1 genes, such as LXRα, was not responsible for the increase (see supplementary data III). These findings were consistent with the previous report that LPS induced the increase of ABCA1 mRNA but not

ABCG1 mRNA in the mouse liver in vivo and in undifferentiated THP-1 cells in vitro (32). Those authors also showed that the induction of ABCA1 expression in THP-1 cells was blocked by PD169316, a p38 mitogen-activated protein kinase inhibitor (32). Another report demonstrated the induction of ABCA1 mRNA by TNF-α through nuclear factor κB (NF-κB) in mouse peritoneal macrophages (33). In contrast, many other reports have stated that ABCA1 is negatively regulated in inflammation. Administration of IL-1β to undifferentiated THP-1 cells (34) and LPS to RAW264 cells (35) resulted in NF-κB activation and ABCA1 suppression. However, none of these reports has yet to find an exact NF-κB binding site(s).

LXR can also be a target of LPS. However, LXRα mRNA was not influenced by the LPS treatment in the mouse liver in our findings (see supplementary data III). LPS downregulated LXRα and ABCA1 in the kidney in mice (36). Repression of LXR by LPS was also found in the hamster liver (37). Lipid A, a component of LPS, but not TNF-α or IL-1β inhibited the LXR ligand-induced ABCA1 expression in peritoneal macrophages in vitro, being mediated through TLR 3/4 and IRF3 (38). Nevertheless, LPS caused neither a reduction of LXR protein nor a decrease of nuclear protein binding to an LXR response element, despite the decrease of ABCA1 and ABCG1 mRNA in J774 cells (39). The regulation of ABCA1 in the acute phase is important for understanding changes in lipid and lipoprotein metabolism in such a condition. Further extensive studies are required for the full elucidation of these findings.

The turnover of ABCA1 protein is rapid, with a half-life of 1–2 h (22, 40, 41). ABCA1 protein in the liver continued to increase until 16 or 24 h after the LPS treatment and remained higher than the control level even at 48 h after treatment (Fig. 4A), whereas the ABCA1 mRNA level returned to normal or even lower after 16 h (Fig. 3). In contrast, both the message and protein levels of ABCG1 remained constant throughout this period (Figs. 3, 4A). Helical apolipoproteins such as apoA-I protect ABCA1 from its proteolytic degradation (22, 40), as do many other amphiphilic helical peptides, including apolipoproteins and synthetic peptides (42). Consistent with those findings, exogenously added lipid-free SAA protected ABCA1 protein from degradation in vitro (Fig. 4). Therefore, an increase of SAA secretion in the liver may likely cause the stabilization of ABCA1 in vivo during acute phase reactions. No such effect was apparent in extrahepatic tissues in LPS-treated animals (Fig. 4C), because helical apolipoproteins stabilize ABCA1 only in their lipid-free forms (22), the liver is the dominant organ in the production of SAA (2), and very little SAA was found in the lipid-free fraction of plasma (27).

HDL is proposed to neutralize LPS (1), and this view may be consistent with a greater increase of plasma TNF-α in HDL-deficient ABCA1-KO mice than in WT mice after LPS treatment (Table 1). Relative induction of SAA in the liver, however, was smaller in spite of a higher plasma TNF-α level in ABCA1-KO mice (Figs. 2B, C, 3). Glucocorticoids are known to enhance SAA induction by cytokines (2), and plasma corticosterone concentration was very low in ABCA1-KO mice even after LPS treatment (data not shown), presumably as a result of the shortage of cholesterol storage in the adrenal glands. This might be the reason for the low response of SAA expression.

Acute phase HDL may also remove cholesterol from cells, although to a lesser extent than normal HDL (43). However, the specific functions of SAA-containing HDL remain unclear. Acute phase SAA is found in all of the vertebrates examined and is highly conserved across evolutionarily distinct species, indicating that the induction of SAA in an acute phase should be one of the fundamental and important reactions to general stress, including inflammation (2). Many reports indicated protective functions of SAA against infection and inflammation, such that SAA binds to the outer membrane protein A of Gram-negative bacteria (44) and acts as an opsonin (45). However, it is unknown whether these SAA functions are related to its presence in HDL. On the other hand, SAA is a precursor of amyloid A, the principal component of the secondary amyloid plaques (2), representing an unbeneficial aspect of its overproduction. As the clearance of lipid-free SAA is more rapid than that of HDL-bound SAA (46), the lipidation of SAA prolongs its life in the circulation and may prevent it from deposit to the tissues. Thus, SAA induction in the ABCA1-deficient condition would aggravate a risk for secondary amyloidosis. Further studies are required to address these questions.

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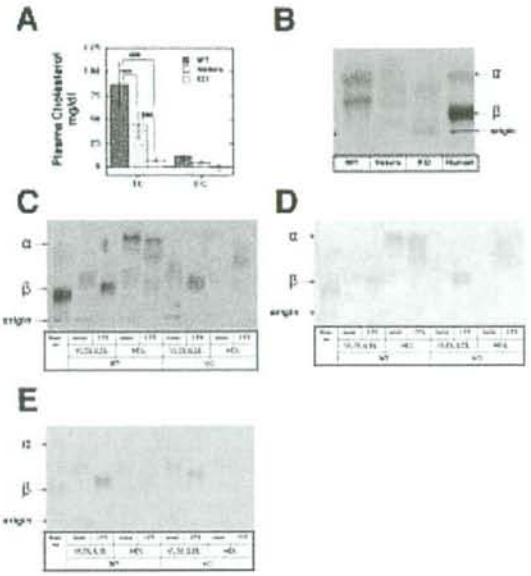
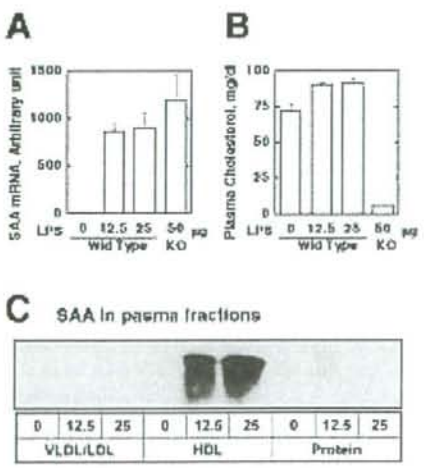
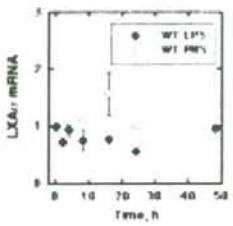
Supplementary Data 1 Effect of the LPS treatment on plasma HDL in mice. (**A**) Plasma lipoprotein lipid concentration at the baseline condition. Total cholesterol (TC) and free cholesterol (FC) of WT (gray columns), ABCA1-hetero (meshed columns) and ABCA1-KO mice (white columns) are shown. Each value represents the mean ± SD (n = 8). Significant statistical difference between the genotypes is indicated as *** ($p<0.001$). (**B**) Plasma of WT, ABCA1-hetero and ABCA1-KO mice were analyzed by agarose gel electrophoresis and by Sudan black staining. Human plasma was also applied as a reference. HDL and LDL in human plasma are indicated as | and |, respectively. (**C, D and E**) Agarose gel electrophoresis of mice plasma after the LPS injection. Plasma was collected from WT mice (WT) and ABCA1-KO mice (KO) at 0 hour (none) and 24 hours (LPS) after LPS injection. VLDL/LDL and HDL fractions were isolated by ultracentrifugation at density below 1.063 g/ml and between 1.063 and 1.21 g/ml, respectively. Each fraction was analyzed by agarose gel electrophoresis, and lipoproteins were visualized by Sudan black staining (**C**), and by specific staining for cholesterol (**D**) and TG (**E**) using an enzymatic colorization method (JR18 and JR19; Helena Laboratories), respectively. VLDL/LDL derived from 0.66 µl of plasma and HDL from 0.33 µl of plasma were analyzed in each lane. HDL and LDL in human plasma are indicated as | and |, respectively.

Supplementary Data 2. Effects of low dose of LPS on WT mice. WT mice were treated with LPS (0, 12.5 and 25 µg). The liver and plasma were collected at 16 hours after injection. A: Liver SAA mRNA determined as Figure 3A. B: Plasma cholesterol concentration determined as described in the text. C: Distribution of SAA among plasma fractions analyzed by Western blotting.

Supplementary Data 3 Time-dependent change of LXRβ mRNA in the liver after injection of LPS in WT mice. The liver was removed and LXRβ mRNA was determined as described in Figure 3. Primers used for quantitative RT-PCR were 5'-TCA ACT GGG GTT GCT TTA GG-3' (forward) and 5'-CTT TTT CCG CTT TTG TGG AC-3' (reversed).







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Reactivity of Astrocytes to Fibroblast Growth Factor-1 for Biogenesis of Apolipoprotein E-High Density Lipoprotein is Down-regulated by Long-time Secondary Culture

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We previously showed that astrocytes produce and release fibroblast growth fator-1 (FGF-1) upon 1-month primary and 1-week secondary culture (M/W cells) and stimulate themselves by an autocrine manner to produce apoE-high-density lipoproteins (HDL), closely associated with their generation of apoE-HDL in brain injury. Astrocytes prepared by 1-week primary and 1-month secondary culture (W/M cells), however, expressed FGF-1 as much as M/W cells but produce apoE-HDL much less. The W/M cells conditioned medium in fact contained FGF-1 activity to stimulate astrocytes prepared by 1-week primary and 1-week-secondary culture (W/W cells). FGF-1 did not stimulate W/M cells for apoE-HDL biogenesis while it stimulated W/W cells. Phosphorylation of Akt, ERK and MEK were induced by FGF-1 in W/W cells but not in W/M cells. Finally, fibroblast growth factor receptor-1 in the membrane decreased in W/M cells in comparison to W/W cells. Interestingly, the reactivity of astrocytes to FGF-1 was recovered when W/M cells were transferred to the tertiary culture of 1 week. We concluded that astrocytes decrease their reactivity to FGF-1 for apoE-HDL biogenesis in certain conditions. The findings indicate astrocyte FGF-1 enhances biogenesis of apoE-HDL also by a paracrine mechanism.

Key words: astrocytes, apolipoprotein E, high-density lipoprotein, cholesterol, fibroblast growth factor-1.

Abbreviations: apoE, apolipoprotein E; HDL, high-density lipoprotein; FGF-1, fibroblast growth factor 1; DPBS, Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; TLC, thin layer chromatography; BSA, bovine serum albumin; FGFR-1, fibroblast growth factor receptor 1.

Astrocytes play many important roles in the brain for maintaining its function. One of those functions is to produce and secrete apolipoprotein E (apoE) and generate high-density lipoprotein (HDL) with the cellular lipid (*1, 2*). ApoE production increases in the brain when it is injured, acutely and perhaps chronically as well (*3–12*). We found that healing of the experimental cryo-injury of the brain was substantially retarded in the apoE-deficient mice (*13*). Production of fibroblast growth factor-1 (FGF-1) was observed in astrocytes in the peri-injury regions 2 days after the injury both in the apoE-deficient and wild-type mice brain. ApoE production increased a few days later in the same regions of the wild-type mouse brain (*13*). *In vitro*, astrocytes produce and release FGF-1 into the medium when prepared as 1-month primary and 1-week secondary culture (M/W cells) (*14*). These cells themselves produce a large amount of apoE-HDL, and anti-FGF-1 antibody prevented this apoE-HDL production, so that increase of apoE-production in the astrocytes thus prepared seemed due to an autocrine reaction of FGF-1 (*15*). The conditioned medium of M/W cells and FGF-1 stimulated the astrocytes prepared in a conventional method as 1-week primary and 1-week secondary culture (W/W cells). Therefore, we hypothesize that FGF-1 is a trigger for astrocytes to stimulate generation of apoE-HDL for recovery of the brain injury by an autocrime mechanism (*15*).

We further investigated the mechanism for FGF-1 to stimulate apoE-HDL production with respect to intracellular signalling. We identified that FGF-1 initiates apoE gene transcription, biosynthesis of cholesterol and other lipid, and secretion of apoE-HDL, independently (*16*). The PI3K/Akt pathway up-regulates apoE-HDL secretion, the MEK/ERK pathway stimulates cholesterol biosynthesis and an unknown pathway enhances apoE transcription.

FGF-1 is produced and released by M/W cells that are kept for 1 month with other neural cells including neurons. Neurons are removed in the secondary culture by the trypsin treatment of the cells in primary culture, so that the astrocytes in the secondary are free from the influence of neurons. Experiment is thus designed to examine whether production of FGF-1 is induced by the influence of other neural cells such as neurons or by the long-time incubation itself. Astrocytes were therefore prepared after 1-week primary culture either by the conventional method of transferring the cells to the secondary culture of 1 week (W/W cells) or by its extension to 1 month (W/M cells).

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FGF-1 was produced and released by W/M cells as well showing that this is independent of co-culture with other neural cells and perhaps due to the long-time culture of astrocytes. In contrast to the findings with M/W cells, however, the reactivity of astrocytes to FGF-1 seemed to be altered in W/M cells. Although apoE-HDL secretion was found somewhat high in comparison to the conventionally prepared W/W cells, W/M cells showed lower apoE-HDL production than M/W cells. We investigated the underlying mechanism for this phenomenon and found that the cells in this condition produce and release FGF-1 so much as M/W cells but they are poorly reactive to FGF-1. Thus, astrocytes produce and release FGF-1 in certain conditions such as under stress but they may remain reactive to FGF-1 or reduce the reactivity to FGF-1 being dependent on their microenvironment. This finding suggests that FGF-1 acts on astrocytes to stimulate apoE-HDL secretion either (or both) by an autocrine or (and) paracrine reaction(s).

MATERIALS AND METHODS

Preparation of Fetal Rat Astrocytes—Astrocytes were prepared from the 17-day-old fetal brain of Wistar rat according to the method previously described (*14–17*). After removal of the meninges, the brain was cut into small pieces and treated with 0.1% trypsin solution in Dulbecco's phosphate buffered saline (DPBS) containing 0.15% glucose (0.1% trypsin/DPBS/G) for 3 min at room temperature. The cell pellet by centrifugation at 1,000 r.p.m. for 3 min was cultured in F-10 medium containing 10% fetal calf serum (FCS) (10% FCS/F-10) at 37°C for 1 week as a primary culture. After treatment with 0.1% trypsin/DPBS/G containing 1 mM ethylenediaminetetra-acetic acid, the cells were cultured in 10% FCS/F-10 for 1 week (W/W cells) or for 4 weeks (W/M cells) as secondary culture (*14*). W/M cells were further transferred to the tertiary culture of 1 week (W/M/W cells). Population of W/M and W/M/W cells were predominant in astrocytes being consistent with that of W/W and M/W cells according to the criteria we previously reported (*14*).

Synthesis of Cellular Lipids—To measure *de novo* synthesis of lipid, astrocytes were incubated with [^{3}H]-acetate (20 μCi/ml) as indicated in each figure legend and washed three times with DPBS. Lipid was extracted from the cells with hexane/isopropanol (3:2, v/v), and radioactivity was counted in cholesterol, sphingomyelin and phosphatidylcholine after separation by thin layer chromatography (TLC) (*15*).

Cellular Lipid Release into the Medium—For standard measurement of cholesterol and other lipid released into medium (*15*), astrocytes were labelled by incubating with [^{3}H]-acetate (20–40 μCi/ml) in 0.1% bovine serum albumin (BSA)/F-10 as indicated in each figure legend. The cells were washed three times with DPBS and incubated in a fresh 0.02% BSA/F-10 for 5 h. The medium was collected and centrifuged at 15,000 r.p.m. for 30 min to remove the cell debris. Lipid was extracted from the medium with chloroform/methanol (2:1, v/v) and analysed by TLC with diethyl ether/benzene/ethanol/acetic acid (200:250:10:1) and chloroform/methanol/acetic acid/water (25:15:4:1) to determine radioactivity of cholesterol, sphingomyelin and phosphatidylcholine.

Analysis of Protein by Western Blotting—The method was described previously (*15*, *16*). The conditioned medium of astrocytes was treated with 10% trichloroacetic acid and centrifugation at 15,000 r.p.m. for 20 min after the cell debris was removed by centrifugation at 15,000 r.p.m. for 30 min. The cells ware treated with 0.02 M Tris–HCl, pH7.4 containing protease inhibitor cocktail for 15 min with 25 times strong agitations for 10 s every 5 min. After removing nuclei by centrifuging at 3,000 r.p.m. for 20 min, the supernatant was centrifuged at 90,000 r.p.m. for 30 min to obtain cytosol and membrane fractions as supernatant and precipitant. Each sample was analysed by 10% SDS–PAGE and immunostained with rabbit anti-rat apoE (a generous gift from Dr Jean Vance, The University of Alberta), a rabbit anti-phosphorylated Akt (Thr-308) antibody (Cell Signaling Technology), a mouse anti-protein kinase B (PKB) α/Akt antibody (BD Transduction Laboratories), a mouse anti-phosphorylated p44/p42 MAP kinase (Thr202/Tyr204) antibody (Cell Signaling Technology), a rabbit anti-p44/42 MAP kinase antibody (Cell Signaling Technology), a rabbit anti-phosphorylated MEK 1/2 (Ser217/221) antibody (Cell Signaling Technology), a rabbit anti-MEK 1/2 antibody (Cell Signaling Technology), a goat anti-FGF-1 antibody (Santa Cluz Biotechnology) and a rabbit anti-Flg [FGF-receptor 1 (FGFR-1)] antibody (Santa Cluz Biotechnology).

Reverse Transcriptase–Polymerase Chain Reaction (RT–PCR)—Total cellular RNA was extracted from rat astrocytes by RNAqueous™ (Ambion), and aliquot of 0.5 μg was reverse-transcribed to cDNA using high-capacity cDNA Reverse Transcription Kits (Applied Biosystems). The cDNA was subjected to PCR by using the DNA probes for rat FGF-1-mRNA, apoE-mRNA, FGFR-1-mRNA [Gene Amp (Applied Biosystems)]. After an electrophoresis of the products, an agarose gel was stained with EtBr solution (Nippon Gene Co. Ltd., Tokyo). The band was detected by an ultraviolet transilluminator (UVP NLM-20 E) at 302 nm. The primer pairs were 5′-AAGCCCGTCGGTGTCCATGG-3′ (sense) and 5′-GATGGCACAGTGGATGGGAC-3′ (anti-sense) for FGF-1, 5′-CTGTTGGTCCCATTGCTGAC-3′ (sense) and 5′-TGTGTGACTTGGGAGCTCTG-3′ (anti-sense) for apoE and 5′-TTGTGGCCTTGACCTCCAAC-3′ (sense) and 5′-TCCCCTGAAGAGCAGGTAGA-3′ (anti-sense) for FGFR-1.

RESULTS

Expression of FGF-1 and apoE in Long-Time Cultured Astrocytes—The message of FGF-1 increased in rat astrocytes in both W/M and M/W cells (prepared by long-time culture, for secondary and primary, respectively), in comparison to the astrocytes by conventional preparation (W/W cells, 1 week for each primary and secondary culture) (Fig. 1A). The message and secretion of apoE also increased in the both cells but the increase was more prominent in M/W cells (Fig. 1B and C). The data with M/W cells are consistent with our previous results that M/W cells produce and release FGF-1 and stimulate

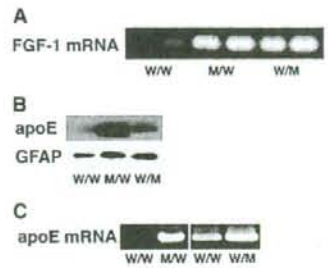

Fig. 1. **Expression of FGF-1 and apoE in astrocytes.** (A) Expression of FGF-1 mRNA in astrocytes prepared by 1-week primary and 1-week secondary culture (W/W), 1-month primary and 1-week secondary culture (M/W) and 1-week primary and 1-month secondary culture (W/M). Total cellular RNA was extracted and subjected for RT–PCR as described in the text. (B) ApoE secreted into the condition media from the W/W, M/W and W/M cells. After preparation, the cells were incubated for 24 h in a fresh 0.02% BSA/F10 medium. Each medium was analysed by western blotting as described in the text. Glial fibrillary acidic protein (GFAP) was also analysed for the cells to indicate equal activation or differentiation of the astrocytes in M/W and W/M cells. (C) Expression of apoE mRNA in astrocytes comparing W/W and M/W cells by RT–PCR. Total cellular RNA, 5 μg, was subjected to reverse transcription, and 0.5 μg of the produced cDNA was amplified by using apoE promer pairs as described in the text, by 26 cycles for W/W and M/W cells, and by 30 cycles for W/W and W/M cells.

the cells for apoE-HDL biogenesis by an autocrine mechanism (*14–16*). ApoE secreted into the medium was all recovered with HDL fraction (d = 1.063–1.21) of the conditioned medium (data not shown), also being consistent with our previous results (*14–16*).

The Conditioned Medium of W/M Cells Stimulates W/W Cells—In order to examine whether FGF-1 is released into the medium in an active form in W/M cells, the conditioned medium was given to W/W cells and biosynthesis and release of cellular lipid was measured. Both biosynthesis and release of lipids were increased in W/W cells by the medium of W/M cells but not by the medium of W/W cells (Fig. 2). The results indicated that the W/M cell-conditioned medium contained FGF-1-like activity, similarly to M/W cells that were examined in our previous reports (*14*, *15*).

The Effects of FGF-1 on W/M Cells—The direct effect of FGF-1 was investigated on W/W cells and W/M cells. Figure 3A shows the effect of FGF-1 on cholesterol biosynthesis. FGF-1 induced increase of cholesterol biosynthesis in a dose-dependent manner in W/W cells and M/W cells, while there was no effect on W/M cells. Figure 3B demonstrates the dose-dependent effect of FGF-1 on W/W and M/W cells for increase of the release of cholesterol and phospholipids, and its no apparent effect on W/M cells for the same parameters. This was reflected in apoE secretion into the medium by astrocytes as it is increased by FGF-1 with W/W cells but not with W/M cells.

Signals Induced by FGF-1 in W/M Cells—FGF-1 was shown to induce signals of the PI3K/Akt pathway for apoE transport and secretion and the MEK/ERK

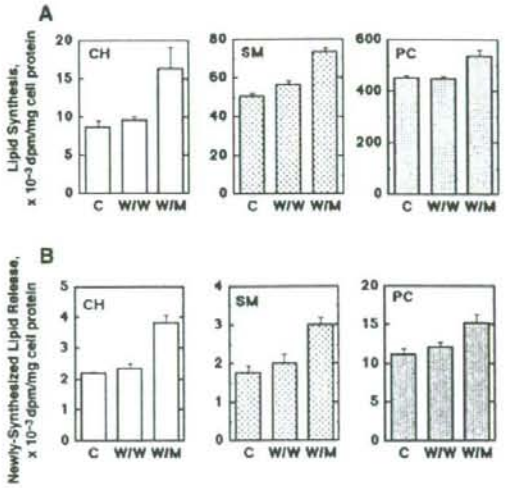

Fig. 2. **Effect of the conditioned medium of W/M cells on rat W/W astrocytes.** (A) Lipid biosynthesis was examined in W/W cells after stimulation by the conditioned medium of W/W and W/M cells. W/W cells were incubated in 500 μl of fresh 0.1% BSA/F-10 plus 500 μl of 0.1% BSA/F-10 or plus 500 μl of the conditioned medium of W/W or W/M cells for 24 h. After washing with DPBS, the cells were incubated with 20 μCi/ml of [^{3}H]-acetate in 1 ml of 0.02% BSA/F-10 for 3 h. The astrocytes were washed three times with DPBS and lipid was extracted from the cells and analysed by TLC to determine radioactivity in cholesterol (CH), sphingomyelin (SM) and phosphatidylcholine (PC) as described in the text. (B) Lipid release from W/W cells was measured. The cells incubated in 500 μl of fresh 0.1% BSA/F-10 plus 500 μl of 0.1% BSA/F-10 or plus 500 μl of the conditioned medium of W/W or W/M cells for 6 h, followed by incubation with 20 μCi/ml of [^{3}H]-acetate for 18 h without washing. After washing with DPBS three times, the cells were further incubated in 0.02% BSA/F-10 for 5 h. Lipid was extracted from the medium and analysed by TLC to determine radioactivity in cholesterol (CH), sphingomyelin (SM) and phosphatidylcholine (PC) as described in the text.

pathway for lipid biosynthesis *via* the FGF receptor(s) (*16*). Therefore, protein phosphorylation was examined for Akt, ERK and MEK proteins upon stimulation of W/W and W/M asctrocytes by FGF-1. Figure 4A shows that each of these signal proteins was phosphorylated by FGF-1 in W/W cells whether present in the membrane or cytosol fractions, while those in W/M cells were not phosphorylated by FGF-1. Finally, the FGFR-1 was analysed for its message and protein. Expression of FGFR-1 mRNA was not different between W/W and W/M cells, and this was reflected by no apparent difference in cytosolic FGFR-1 (Fig. 4B). However, the FGFR-1 protein in membrane significantly decreased in W/M cells in comparison to W/W cells, demonstrated also in Fig. 4B. Molecular weight of the membrane FGFR-1 was similar to that of the larger molecule of the duplex bands of cytosolic FGFR-1, presumably indicating maturation of the receptor protein.

Recovery of the Reactivity of Astrocytes to FGF-1—Finally, the reactivity of astrocytes to FGF-1 was recovered by transferring the W/M cells to the tertiary

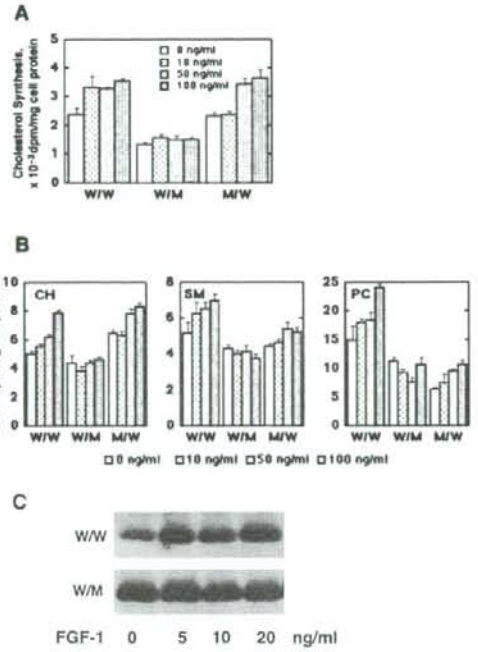

Fig. 3. **Effect of FGF-1 on astrocytes.** (A) W/W, W/M and M/W cells were incubated with FGF-1 (0, 10, 50 and 100 ng/ml) in 0.1% BSA/F-10 for 24 h. The cells were washed and labelled with 20 μCi/ml of [^{3}H]-acetate in a fresh 0.02% BSA/F-10 for 2 h. After washing, lipid was extracted from the cells and radioactivity in cholesterol was analysed by TLC as described in the text. (B) Cellular lipid release by the W/W, W/M and M/W astrocytes. The cells were incubated with the indicated amount of FGF-1 as above and [^{3}H]-acetate as described for 3 h. After washing with DPBS three times, the cells were further incubated in 0.02% BSA/F-10 for 5 h. Lipid was extracted from the conditioned medium after removal of cell debris to analyse by TLC for cholesterol (CH), sphingomyelin (SM) and phosphatidylcholine (PC). (C) Stimulation of apoE secretion by FGF-1. W/W cells and W/M cells were incubated with various amount of FGF-1 as described above for 24 h. After washing and incubation of the cells with 0.02% BSA/F-10 for 24 h, the conditioned medium was analysed for apoE secretion by western blotting.

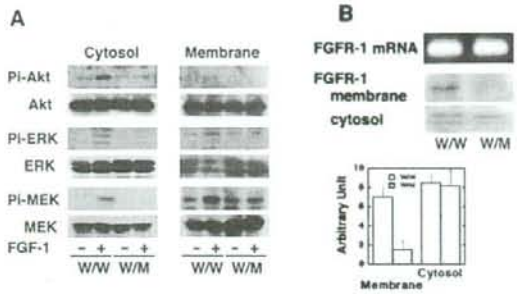

Fig. 4. **Analysis of the signalling pathways in rat astrocytes.** (A) Phosphorylation of signal-related proteins in W/W and W/M cells. The cells were washed and incubated in 0.1% BSA/F-10 for 16 h. After washing, the cells were treated with FGF-1 (50 ng/ml) for 5 min, and the cytosol and membrane fractions were prepared and analysed by 10% SDS–PAGE and western blotting as described in the text by using antibodies against Akt, ERK and MEK proteins and phosphorylated form (Pi) of each of these proteins. (B) Analysis of FGFR-1. Expression of FGFR-1 mRNA in W/W and W/M cells was analysed by RT–PCR for 30 cycles. FGFR-1 protein was also analysed in the membrane and cytosol fractions of W/W and W/M cells by western blotting using specific antibody. The graph indicates the results of scanning of the western blotting gels as mean ± SE for three independent experiments. For cytosolic protein, both bands were analysed together.

culture for 1 week (W/M/W cells). Figure 5A shows induction of phosphorylation of Akt, MEK and ERK by FGF-1 in W/W and W/M/W cells but not in W/M cells. Figure 5B and C show the same change in reactivity to FGF-1 of the lipid biosynthesis and the apoE release.

DISCUSSION

Cholesterol homoeostasis in animals involves intra- and extra-cellular regulation of its metabolism (*18*) and extracellular transport of cholesterol in vertebrates is carried by plasma lipoprotein system. However, the blood–brain barrier prevents central nervous system from accessing to this system, so that it operates a unique and independent specific lipoprotein system for extra-cellular

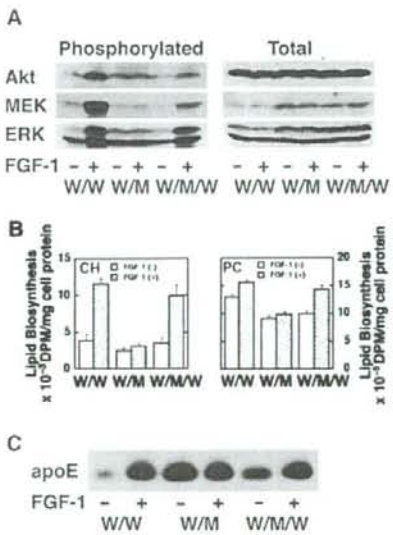

Fig. 5. **Recovery of reactivity of astrocytes to FGF-1 by short tertiary culture.** W/M cells were transferred to the tertiary culture of 1 week and analysed for the reactivity to FGF-1. The experiments were conducted in the same manner as those in Figs 3 and 4. (A) Phosphorylation of signalling proteins induced by FGF-1. (B) Increase of biosynthesis of cholesterol and phosphatidylcholine. (C) Secretion of apoE into the medium.

cholesterol transport. HDL is an exclusively found lipoprotein in cerebrospinal fluid that contains mainly apoE and apoA-I (*19*). While apoA-I is not synthesized by neural cells and its origin is uncertain (*20, 21*), apoE is known to be synthesized at least in astrocytes and microglias to generate apoE-HDL (*1, 2*). Many reports suggest that apoE-HDL delivers cholesterol to neurons and this lipoprotein seems to increase in the lesions of injury of brain and nerves (*3–12*).

We have reported that FGF-1 is a key factor for astrocytes to produce apoE-HDL in response to damage of nerve system, perhaps for both acute and chronic, major and minor injuries. In mouse brain, production of FGF-1 by the astrocytes in the peri-injury regions is observed prior to the appearance of apoE in the same cells, and the lack of apoE production retarded the recovery of the injury (*13*). Being consistent with this finding, astrocytes prepared by long primary culture and 1-week secondary culture (M/W cells) produce and release FGF-1, which stimulates these astrocytes themselves in an autocrine manner for production of apoE-HDL (*14, 15*). FGF-1 also stimulates the astrocytes prepared by a conventional method of 1-week primary and 1-week secondary cultures (*15*).

When the secondary culture was prolonged for 1 month (W/M cells), the astrocytes produced apoE-HDL more than conventionally prepared W/W cells, but much less than M/W cells. However, the conditioned medium contained the activity to stimulate W/W cells for production of apoE-HDL and related reactions such as increase of lipid biosynthesis. W/M cells showed the loss of their reactivity to FGF-1 including signals activated *via* the FGFR-1 (*16*) and this seemed due to the decrease of FGFR-1 in the membrane fraction.

An exact reason and mechanism for this change are not known. Long-time primary culture does not induce such change in astrocytes, and long secondary culture resulted in loss of the reactivity of the cells to FGF-1. The primary culture cell population contains many types of cells other than astrocytes such as neurons, oligodendroglias and microglias at least at the beginning of the culture, and neurons gradually disappear in the 3-week primary culture before the cells are transferred to the secondary culture. The secondary culture starts after loss or decrease of the other types of cells almost exclusively with astrocytes. Thus, the presence of other types of neural cells for the long-time culture was one of the potential reasons to protect astrocytes from loss of the cellular reactivity to FGF-1. However, the finding that transferring the cells to the tertiary culture recovered the reactivity to FGF-1 excluded such possibility. This result rather indicated that the difference in microenvironment in relation to stage of cellular proliferation might cause some specific change in astrocytes with respect to expression of FGFR-1.

Although an exact mechanism for astrocytes to lose their FGF-1-reactivity is unknown, we cannot exclude the possibility that such change may be induced also *in vivo* when the astrocytes are exposed to a kind of specific stress. If this takes place, FGF-1 induces apoE-HDL biogenesis and excretion not only by autocrine reactions, but also by paracrine reactions with healthy astrocytes.

It is thus important to carry out further investigation on the mechanism by which the brain injury triggers production and release of FGF-1 by astrocytes in the peri-injury regions, and how FGF-1 stimulates the astrocytes in the regions. This process is a key for the recovery of the grain from the injury, so that the detailed information for the mechanism of these reactions will provide the base of development of technology to expedite this healing process.

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ApoA-I Facilitates ABCA1 Recycle/Accumulation to Cell Surface by Inhibiting Its Intracellular Degradation and Increases HDL Generation

Rui Lu, Reijiro Arakawa, Chisato Ito-Osumi, Noriyuki Iwamoto and Shinji Yokoyama

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ApoA-I Facilitates ABCA1 Recycle/Accumulation to Cell Surface by Inhibiting Its Intracellular Degradation and Increases HDL Generation

Rui Lu, Reijiro Arakawa, Chisato Ito-Osumi, Noriyuki Iwamoto, Shinji Yokoyama

Objective—Calpain-mediated proteolysis is one of the major regulatory factors for activity of ATP-binding cassette transporter (ABC) A1. Helical apolipoproteins protect ABCA1 against this degradation and increase generation of HDL. We investigated the mechanism for this reaction focusing on roles of endocytotic internalization of ABCA1.

Methods and Results—Surface ABCA1 was labeled with biotin and traced for its internalization and degradation. ABCA1 in the cell surface was internalized within 10 minutes regardless of the presence of apoA-I. ABCA1 was intracellularly degraded and was protected against this only when exposed to extracellular apoA-I before its endocytosis. Consequently, recycle of ABCA1 to the surface was enhanced, and surface ABCA1 was increased by apoA-I. Direct inhibition of ABCA1 endocytosis led to decrease of its degradation and increase of surface ABCA1. Generation of HDL increased in parallel with surface ABCA1.

Conclusion—Surface ABCA1 is internalized and degraded, and apoA-I interferes with only the latter step to recycle ABCA1 to the surface. Increase of surface ABCA1 results in the increase of generation of HDL. **(*Arterioscler Thromb Vasc Biol.* 2008;28:1820-1824)**

Key Words: ABCA1 ■ calpain ■ HDL ■ endocytosis ■ apoA-I ■ cholesterol

High density lipoprotein (HDL) is biogenerated with helical apolipoproteins and cellular lipid being mediated by the membrane protein ATP-binding cassette transporter (ABC) A1.[1] Helical apolipoprotein, mainly apolipoprotein A-I (apoA-I), is delivered and liberated by plasma HDL to somatic cells that do not synthesize apolipoproteins for biogenesis of new HDL,[2] whereas apoA-I is likely secreted from hepatocytes in a free form and interacts with its own ABCA1 in an autocrine manner.[3] HDL particles are formed with apolipoprotein and membrane phospholipid, and cholesterol is integrated into this particle being dependent on various cellular factors.[4,5] ABCA1 expression is upregulated mainly by the liver X receptor as sensing a cellular oxysterol level,[6] but it is also under the regulation by sterol regulatory element binding protein 2 in the liver perhaps to properly maintain whole body cholesterol homeostasis.[7] Interestingly, it is downregulated by activator protein 2α,[8] but its physiological role is unknown. On the other hand, ABCA1 is rapidly degraded by calpain and it seems an important regulation system for its activity of generation of HDL.[9] This proteolytic degradation is retarded when ABCA1 interacting with helical apolipoproteins[9,10] suggesting that this is a positive feedback system for HDL biogenesis. This must be a steady state ongoing in vivo in most of the cells that are chronically exposed to helical apolipoproteins such as apoA-I, although this view is yet to be proven for its relevance. It was proposed that ABCA1 is internalized by endocytosis and seems recycled,[11,12] and HDL biogenesis is associated with such endocytotic reactions.[13–16] Involvement of adaptor proteins is also suggested in the endocytosis and degradation of ABCA1.[17,18] Deletion of PEST sequence of ABCA1 inhibited its endocytosis, degradation by calpain, and HDL biogenesis, suggesting that endocytosis of ABCA1 is a key process for these all.[19] However, there are other views that HDL biogenesis takes place rather in the cell surface[20–22] than in the endosomes, where ABCA1 is entrapped. Most of these studies were carried out with ABCA1 transfected and overexpressed. In this work, we attempted to understand this complicated process by labeling the endogenous ABCA1 in cell surface and tracing it.

Materials and Methods

Cell Culture and ApoA-I

BALB/3T3 clone A31 cells were maintained and incubated in 5% Eagle minimum essential medium (MEM) with low glucose and 30% Ham F-12 (Wako) with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2.[23] THP1 cells were differentiated to macrophages by incubating in 10% FCS in the presence of 3.2×10^{-7} mol/L of phorbol 12-myristate 13-acetate (PMA; Wako)

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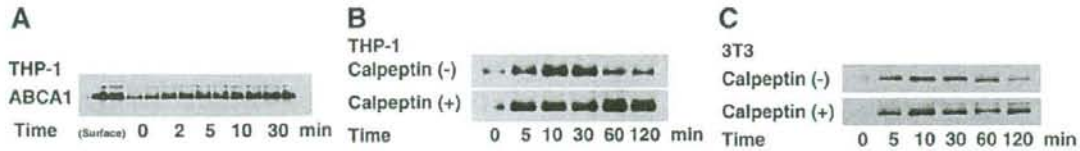

Figure 1. Internalization of ABCA1. A, Cell surface protein of the differentiated THP-1 cells was pulse-labeled with sulfo-SS-biotin. After further incubation at 37°C for the indicated time, surface biotin was cleaved to detect ABCA1 internalized. Biotinylated protein was selectively adsorbed by streptavidine-agarose and analyzed by Western blotting for ABCA1. Surface ABCA1 indicates the samples just after the biotinylation. B, Internalized-ABCA1 was detected in the presence or absence of 100 μmol/L of calpeptin in the same condition as in A. C, Internalization of ABCA1 was analyzed in BALB/3T3 cells by the same procedure as described above.

for 24 hours.[23] Human apoA-I was isolated from human plasma HDL as described previously.[24] It was used at 10 μg/mL in the medium for all the experiments unless otherwise specified.

Labeling and Tracing ABCA1

Cell surface proteins were biotinylated with sulfosuccinimidyl 2-(biotinamido)-rthyl-1, 3-dithiopropionate (sulfo-SS-biotin) (Pierce) for 1 hour at 4°C according to the methods previously reported.[25] After quenching the reaction, cells were washed and lysed, and the membrane fraction was prepared as described previously.[9] Biotinylated membrane proteins were isolated by incubating with streptavidin-agarose beads (Sigma) at 4°C for 1 hour.[26] After recovering the beads, proteins bound to the beads were eluted by incubating in the sample buffer for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then analyzed for Western immunoblotting by using specific antibody against ABCA1 as previously described.[9] To trace the labeled surface ABCA1 for internalization, biotinylation of the cell surface proteins was cleaved by incubating the cells with 50 mmol/L reduced glutathione (Sigma) in pH 7.8 three times for 20 minutes,[26] and the remaining biotinylated ABCA1 was analyzed as above as the internalized portion. Intracellular ABCA1 degradation was measured as the time-dependent decrease of biotinylated ABCA1 after the surface biotin was cleaved after the incubation of the biotinylated cells for 1 hour at 37°C. To examine recycle of ABCA1, intracellular ABCA1 was prelabeled as above. At the various period of the incubation, the cell surface biotin was cleaved again and the remaining biotinylated ABCA1 was analyzed and compared with the biotinylated ABCA1 without the second cleavage to estimate the resurfaced ABCA1.

Cellular Lipid Release

Cellular lipid release by apoA-I was measured as described elsewhere. After incubation of the cells with apoA-I for the indicated time, concentration of cholesterol and choline-phospholipid in the medium were evaluated by enzymatic measurement.[27]

Quantification of Western Blotting Results

The bands were digitally scanned by using an EPSON GT-X700 and analyzed with Adobe Photoshop software.

Results

Figure 1A shows time-dependent internalization of ABCA1 in THP-1 cells. Most of the biotinylated ABCA1 in the surface was recovered as the internalized protein after incubating the cells at 37°C longer than 10 minutes, by cleaving the surface biotinylation with glutathione after the incubation. The internalized ABCA1 apparently decreased after 30 minutes of incubation, and this decrease was inhibited by a calpain inhibitor, calpeptin, both in THP-1 cells and BALB/3T3 cells (Figure 1B and 1C). ABCA1 was thus shown degraded by calpain after the internalization.

Degradation of ABCA1 by calpain was shown inhibited by helical apolipoproteins, such as apoA-I.[9] Therefore, the internalization of ABCA1 was examined in the presence of apoA-I. After 10 minutes of the incubation, most of the surface-labeled ABCA1 was internalized regardless of the presence of apoA-I (Figure 2A). This process was not modified any further even by the presence of calpeptin, indicating that ABCA1 was protected by apoA-I against the calpain-mediated degradation (Figure 2B). To investigate whether ABCA1 is "preprotected" by apoA-I before its internalization or extracellular apoA-I protects ABCA1 even after it is internalized, degradation of the internalized ABCA1 was examined for timing of adding apoA-I (Figure 2C). When apoA-I was present in the medium for the period before the internalization of the prebiotinylated surface ABCA1, degradation of ABCA1 was retarded (apoA-I (+)). However, when apoA-I was added to the medium after the prelabeled ABCA1 was internalized, the degradation was not much retarded (Chased). This result indicates that the protective effect of apoA-I on ABCA1 against its degradation is achieved before ABCA1 is internalized, and not by cell–apoA-I interaction to cause a distant effect on the internalized ABCA1.

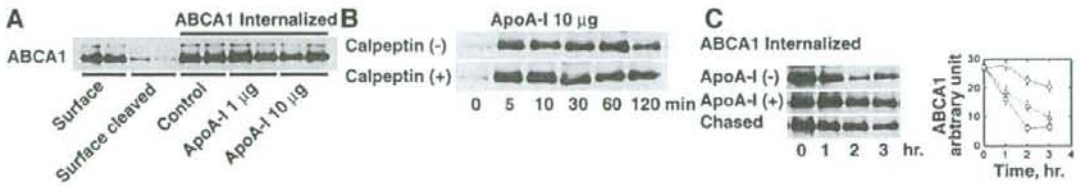

Figure 2. ABCA1 internalization and the effect of apoA-I in BALB/3T3 cells. A, ABCA1 internalization in the presence of apoA-I for 10 minutes at 37°C. "Surface" ABCA1, immediately after the labeling. "Surface cleaved", after biotin cleavage before internalization. B, ABCA1 internalization with apoA-I with and without calpeptin. C, Retardation of ABCA1 internalization by apoA-I. Cells were biotinylated and incubated for 1 hour at 37°C, and surface biotinylation was cleaved. Biotinylated ABCA1 was analyzed by Western blotting. ApoA-I (−) indicates without apoA-I throughout the incubation (squares); ApoA-I(+), with apoA-I in the preinternalization period (circles); Chased, apoA-I was added after the surface biotin cleavage (triangles).

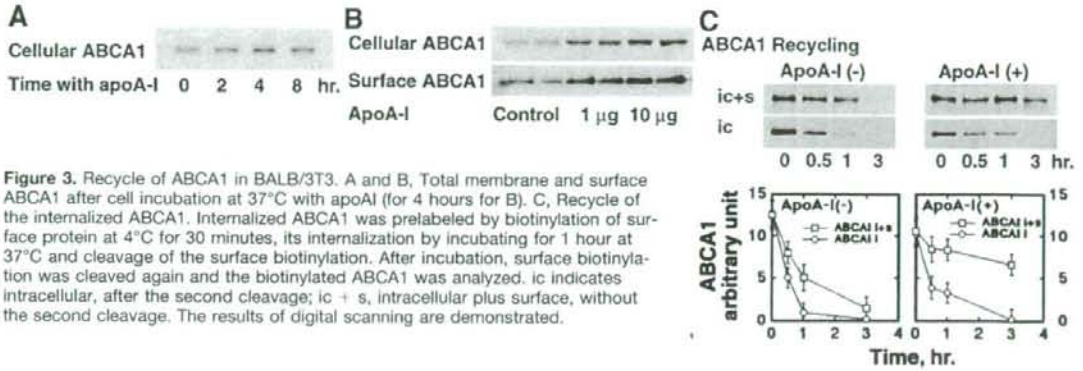

Figure 3. Recycle of ABCA1 in BALB/3T3. A and B, Total membrane and surface ABCA1 after cell incubation at 37°C with apoAI (for 4 hours for B). C, Recycle of the internalized ABCA1. Internalized ABCA1 was prelabeled by biotinylation of surface protein at 4°C for 30 minutes, its internalization by incubating for 1 hour at 37°C and cleavage of the surface biotinylation. After incubation, surface biotinylation was cleaved again and the biotinylated ABCA1 was analyzed. ic indicates intracellular, after the second cleavage; ic + s, intracellular plus surface, without the second cleavage. The results of digital scanning are demonstrated.

ABCA1 in the whole cell membrane increased up to 4 hours of the incubation, and this increase was parallel between the whole cell and cell surface (Figure 3A and 3B). To examine the mechanism for this increase of the surface ABCA1, recycle to the surface of the internalized ABCA1 was examined. After the prelabeled ABCA1 was internalized and surface biotinylation was cleaved, the cells were further incubated for certain periods of time and the surface biotinylation was cleaved again to assess the recycled ABCA1 to the surface (Figure 3C). In the absence of apoA-I, the internalized ABCA1 rapidly disappeared, and only its small portion was found recycled to the surface. In the presence of apoA-I, clearance of the internalized ABCA1 was substantially retarded as presented above, and a large portion of it was found recycled to the surface. Thus, apoA-I increased recycling of ABCA1 apparently by blocking the intracellular calpain-mediated degradation.

To examine whether internalization of ABCA1 is mandatory for the HDL biogenesis reaction, clathrin-mediated endocytosis was inhibited by cytochalasin D.[28,29] ABCA1 in the cell was decreased within 60 minutes in the absence of helical apolipoproteins when its synthesis was inhibited by cycloheximide (Figure 4A). When the endocytosis was inhibited by cytochalasin D, ABCA1 did not decrease. The increase of cellular ABCA1 by cytochalasin D was shown attributable to its increase in the cell surface (Figure 4B) as its endocytosis was strongly inhibited (Figure 4C).

Finally, generation of HDL was evaluated by measuring release of cellular phospholipid and cholesterol by apoA-I[5,30] when the endocytosis of ABCA1 was inhibited and its amount in the cell surface was increased. As shown in Figure 5, releases of phospholipid and cholesterol were both increased by this treatment. As apoA-I by itself increases surface ABCA1 by increasing its recycling, the increment of the HDL biogenesis should not be to the same extent as the increase of surface ABCA1 by cytochalasin D in the absence of apoA-I. This was in fact demonstrated in Figure 5B. The relative increase of the surface ABCA1 by cytochalasin D was to a less extent in the presence of apoA-I because the surface ABCA1 is already increased by apoA-I even in the absence of cytochalasin D. The increase of ABCA1 by cytochalasin D in the presence of apoA-I was parallel to the increase of lipid release by apoA-I (Figure 5C).

Discussion

In summary, we have shown that: (1) ABCA1 is rapidly degraded intracellularly by calpain after its clathrin-mediated endocytotic internalization in the absence of helical apolipoprotein; (2) Helical apolipoproteins, represented by apoA-I, protect ABCA1 from the degradation, not by inhibiting the internalization but by inhibiting the intracellular proteolysis; (3) This inhibition is achieved by preexposure of ABCA1 to extracellular apoA-I before the internalization and not by the exposure of the cells after ABCA1 is internalized;

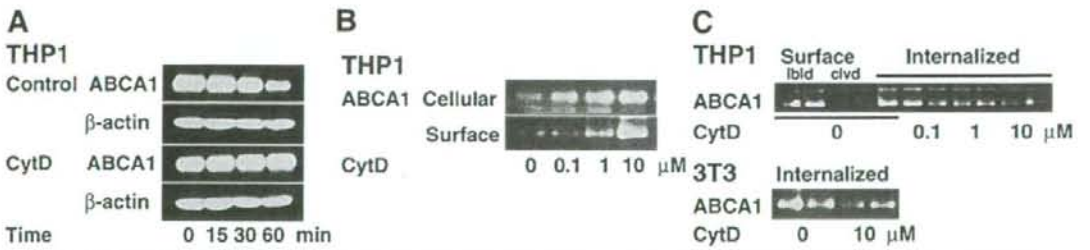

Figure 4. Effects of cytochalasin D on ABCA1 in differentiated THP-1 cells. A, Cells were incubated with 10 μmol/L cytochalasin D at 37°C and total membrane ABCA1 was analyzed. B, ABCA1 in total membrane and cell surface after incubation with cytochalasin D at 37°C for 30 minutes. C, ABCA1 internalization in the presence of cytochalasin D. Surface Ibld indicates the biotinylated ABCA1 immediately after the labeling; Surface clvd, biotinylated ABCA1 after cleavage of surface biotin before the incubation at 37°C; Internalized, ABCA1 after the incubation at 37°C for 10 minutes and cleavage of the surface biotin. ABCA1 in BALB/3T3 cells were also analyzed for the internalization by the incubation for 10 minutes at 37°C.

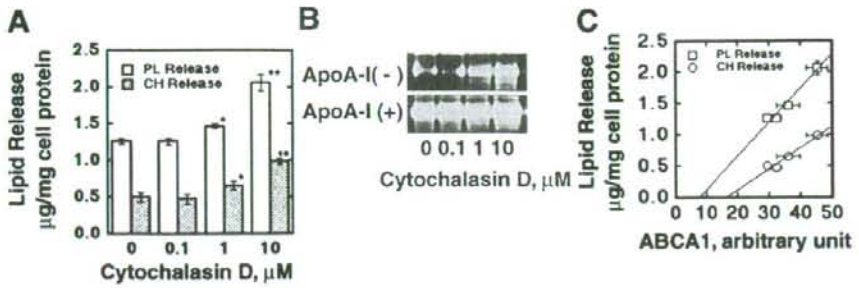

Figure 5. Effect of cytochalasin D on the apoAI-mediated HDL biogenesis. A, Differentiated THP-1 cells were incubated with apoA-I for 6 hours, and release of cholesterol and phospholipids was measured. The effect of cytochalasin D was examined in a dose-dependent manner. The data represent mean± SE for 3 measurements as $^{*}P<0.05$ and $^{**}P<0.01$ from the control. B, ABCA1 protein in cell surface in the absence and presence of apoA-I for 6 hours. C, The results of B were digitally scanned, and the lipid release data were plotted against the surface ABCA1.

(4) ABCA1 that escaped from the intracellular proteolysis is recycled to the cell surface, and apoA-I therefore enhances this process to increase cell surface ABCA1; (5) Generation of HDL is directly proportional to the surface ABCA1 level. The results are summarized in supplemental Figure I (available online at http://atvb.ahajournals.org).

It is well recognized that activity of ABCA1 is a rate-limiting factor for biogenesis of HDL and therefore plasma HDL concentration in vivo.[1] Expression of the gene has been shown to regulate it in vitro and in vivo,[6–8] but the degradation of ABCA1 protein seems an important regulatory factor for its activity as a posttranslational regulation at the cellular level,[9,10] whose physiological relevance, however, is yet to be proven.

We used THP-1 cells and BALB/3T3 fibroblasts as models for generation of HDL. HDL biogenesis in vivo is largely in the liver and intestine,[31] but any peripheral cells must carry on the HDL biogenesis reaction for their cholesterol homeostasis.[1] Indeed, it was proposed that peripheral tissue may be a significant source of plasma HDL in human.[32] Therefore, the use of these cells is justified to investigate mechanism for HDL biogenesis by the ABCA1/apolipoprotein system. ABCA1 seems stabilized in hepatocytes in an autocrine mechanism by a large amount of apoA-I produced and secreted by themselves, and the effects of additional apoA-I may not be apparent.[3]

When HDL generation is ongoing, helical apolipoproteins interact with ABCA1 before its internalization and make ABCA1 resistant to the calpain-mediated degradation. Consequently, a large portion of ABCA1 is recycled to the surface without degradation for further HDL generation. This view is consistent with most of the previous findings that apoA-I/ABCA1 complex recycles and apoA-I may be released by exocytosis during the HDL generation reactions.[11,12] Most of the cells in the body are chronically exposed to HDL, which liberates apoA-I for generation of HDL.[2] Therefore, in the physiological environment in vivo, ABCA1 seems protected from the degradation and its clearance rate should be rather slow. Recently, 2 independent articles proposed that HDL biogenesis by ABCA1 mainly takes place on cell surface rather than in the endosomes by tracing the labeled apoA-I in the presence of transfected ABCA1.[21,22] The conclusion in the present work is consistent with these proposals and may not agree with the view that HDL biogenesis occurs intracellularly.[13–16]

In the steady state of HDL generation, ABCA1 should be increased in cell surface from the baseline condition without apolipoprotein. It is therefore of interest whether there is a room for further increase of surface ABCA1 and consequently for the increase of HDL biogenesis by inhibiting the endocytotic internalization of ABCA1. Inhibition of the endocytosis by cytochalasin D increased surface ABCA1×50 to 60% as well as HDL biogenesis in parallel (Figure 5C).

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Disclosures

Shinji Yokoyama is involved in establishment of a Venture Company, HYKES Laboratories.

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