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consequently in a disc-like shape. The question then becomes how and where these particles are formed. If the apolipoprotein-cell interaction is a major mechanism for production of HDL, it is possible that HDL is assembled by an autocrine mechanism, such that apoA-I or E are first secreted by the cells and then interact with the cell surface to generate HDL (51,52). This hypothesis has more directly been supported by using an ABCA1 inhibitor, probucol, and the above-mentioned antibody specific to lipid-free apoA-I to inhibit ABCA1-dependent HDL assembly by hepatocytes (53). When HepG2 cells were treated with probucol, apoA-I otherwise found associated with HDL was secreted all in a free form (Figure 4A). In the presence of the antibody, generation of HDL was completely inhibited (Figure 4BC) while it did not influence the pre-produced HDL in the medium.

Thus, lipid-free apolipoprotein is to be released whether from cells or from HDL particles to interact with cellular ABCA1 for assembly of HDL particles from cellular lipid. Alternatively, apolipoproteins may interact in part with the membrane already somewhere before the secretion through the same mechanism as extracellular apolipoprotein reacts (54,55). This view may be consistent with the finding of the abnormal Golgi structure in the hepatocytes of ABCA1 knock-out mice (34) and differential generation of HDL with endogenous apoE and exogenous apoA-I by rat astrocytes (56).

Assembly of HDL particles and cholesterol enrichment

Apolipoprotein recruits primarily phospholipid rather than cholesterol to form stable HDL particles in this

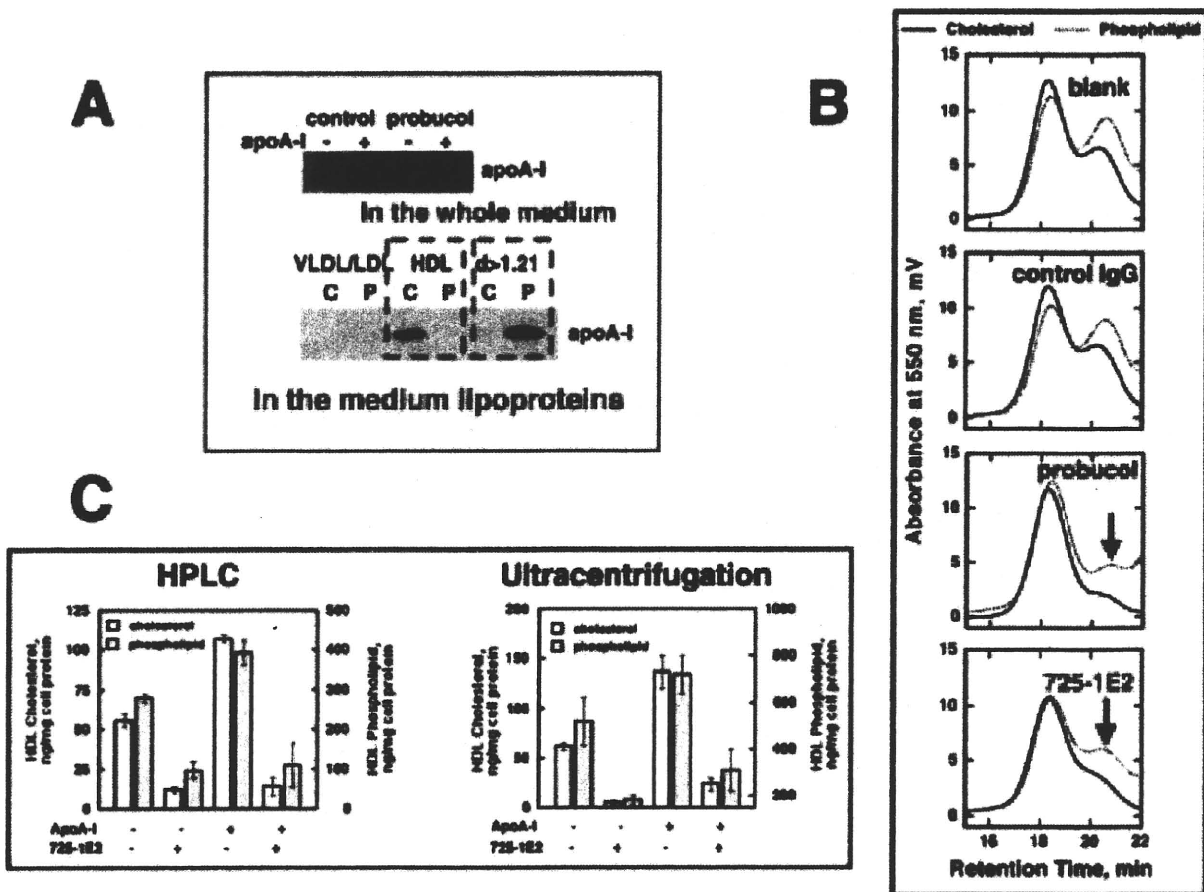

Figure 4. Biogenesis of HDL by HepG2 cells with endogenous apoA-I (53). Panel A shows the results of apoA-I secretion when HepG2 cells were treated with an ABCA1 inhibitor, probucol (P), in comparison to control (C). Secretion of apoA-I into the medium is unchanged by the treatment of the cells with probucol, but apoA-I was recovered all in a lipid-free form by the treatment while it was otherwise all bound to HDL. Panel B shows marked decrease of HDL production by HepG2 cells when ABCA1 was inactivated by probucol or the monoclonal antibody specific to lipid-free apoA-I, 725-1E2, was present in the medium, demonstrated as HPLC profiles of the media. Solid lines indicate cholesterol, and dotted lines indicate choline-phospholipid. Panel C demonstrates the same results shown as quantitative data by using the HPLC analysis data and the ultracentrifugation analysis data.

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HDL assembly pathway (57). HDL generated by this reaction contains largely phospholipid and unesterified cholesterol, and the LCAT-mediated cholesterol esterification on the generated HDL perhaps helps the maturation of this HDL as it generates core cholesteryl ester (11,21). However, unlike cholesterol release by non-specific diffusion-mediated reaction, cholesterol esterification does not result in further enhancement of cellular cholesterol release when the HDL generated is already cholesterol-rich (11).

HDL-like particles can be formed *in vitro* with helical apolipoproteins and phospholipid, with or without core lipid and cholesterol, without specific catalysts except for the requirement of energy for dispersion of the components to homogeneity (58). The reaction always yields the particles of certain sizes composed of at least a few hundreds of phospholipid molecules. Therefore, HDL-like particles are a thermodynamically stable molecular assembly for helical apolipoproteins and phospholipid. The physicochemical nature of apolipoprotein-phospholipid interaction is that 'lipidation' of apolipoprotein takes place primarily with phospholipid in a kind of snap-in manner rather than 'gradual growth'. On the other hand, apolipoprotein cannot form a complex with cholesterol alone. When apolipoprotein interacts with cells through ABCA1, the same type of reaction should take place to generate HDL. In fact, disc-like HDL particles are generated primarily with membrane phospholipid when apoA-I interacts with the cells in the presence of ABCA1 (Figure 5A). However, it has not yet been evident whether premature HDL particles found in plasma are produced by this reaction and are direct precursors of plasma HDL, such as preβ-HDL, γ-LpE, and LpA-IV (59). It should be noted that Miller and colleagues suggested that preβ-HDL in human peripheral tissue fluid should be considered substantially produced locally rather than filtered from blood plasma (60). This finding may support the view that at least apoA-I locally dissociates from HDL and produces new preβ-HDL by removing lipid from peripheral cells.

It was recently reported that ABCA7 also mediates the HDL assembly *in vitro* in a similar manner to ABCA1 when transfected and over-expressed in HEK293 cells (61–63). Analysis of the HDL products by size exclusion high-performance liquid chromatography (HPLC) revealed that ABCA1 generates two different types of HDL, large cholesterol-rich and small cholesterol-poor, while ABCA7 produces only small and cholesterol-poor HDL (Figure 5B) (63,64). Although this reaction may not significantly contribute to the regulation of plasma HDL concentration (65) and the expression of the ABCA7 gene is not regulated for the HDL biogenesis (66), it is still of interest to examine the difference between the two ABC proteins in order to elucidate the mechanism for ABCA1 to remove cellular cholesterol more efficiently in the HDL biogenesis.

Closing remarks

The finding of the mutation in ABCA1 opened a new gate for studying cellular cholesterol homeostasis with respect to its releasing mechanism. This

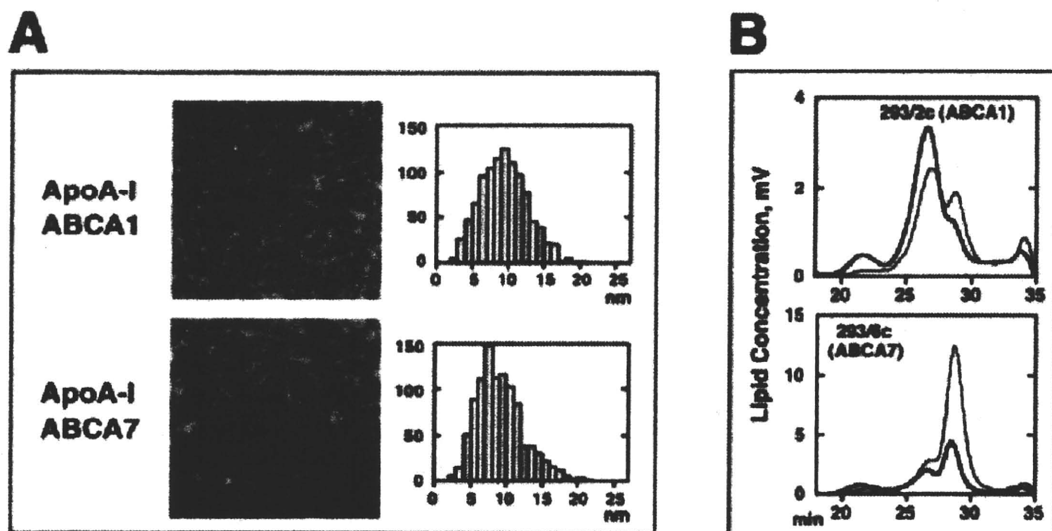

Figure 5. HDL particles generated by apoA-I when ABCA1 or ABCA7 are transfected and over-expressed in HEK293 cells (64). Panel A shows electron microgram of the particles isolated from the medium by ultracentrifugation. Scale bars indicate 100 nm. Histograms represent the results of the measurement of diameters. Panel B shows the results of the HPLC analysis. Thick solid lines represent cholesterol, and thin solid lines represent choline-phospholipid.

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protein undoubtedly plays an essential role in apolipoprotein-mediated assembly of HDL. It is, however, still unclear how ABCA1 functions to mediate the interaction of helical apolipoprotein with phospholipid in the cell membrane. In order to maintain cholesterol homeostasis, diffusion-mediated physicochemical cholesterol release functions as much as the apolipoprotein-mediated pathway both at the cellular level and for the whole body. Therefore, Tangier patients may not develop general and massive cholesterol accumulation since the diffusion-mediated system is preserved (67). This is the same in LCAT deficiency patients who lack a driving force for the net cholesterol release by the diffusion-mediated system but not the apolipoprotein-mediated reaction (68). Thus, the two systems back up each other to maintain cellular and body cholesterol homeostasis (69).

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

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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.**

Supplementary key words serum amyloid A • high density lipoprotein • ATP binding cassette transporter A1 • cholesterol

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

Manuscript received 6 September 2007 and in revised form 15 November 2007 and in re-revised form 21 November 2007.

Published, JLR Papers in Press, November 21, 2007.
DOI 10.1194/jlr.M700402-JLR200

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 CNFAKDQSDDDHLKDLSLHKN, 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 I), 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

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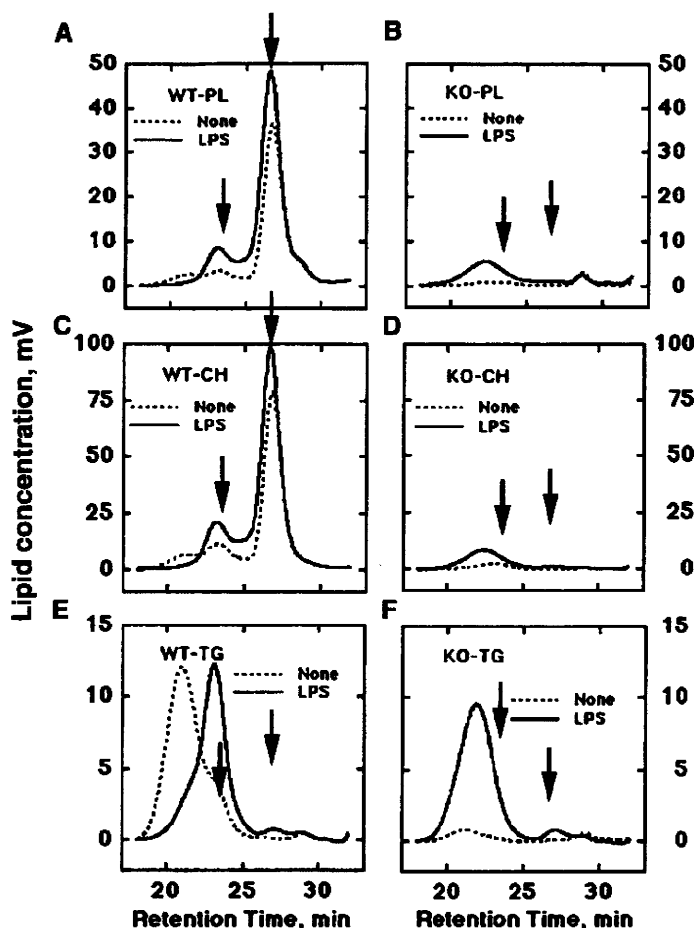

Fig. 1. Effect of lipopolysaccharide (LPS) treatment on mouse plasma lipoprotein. Wild-type (WT) and ABCA1-knockout (KO) mice were treated with (solid lines) and without (dashed lines) LPS. Plasma was collected at 24 h after the treatment and analyzed by molecular sieve HPLC. A and B represent monitoring 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

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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[a]	0.55 ± 0.31[a]	1.48 ± 0.33[a,d,e]
	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

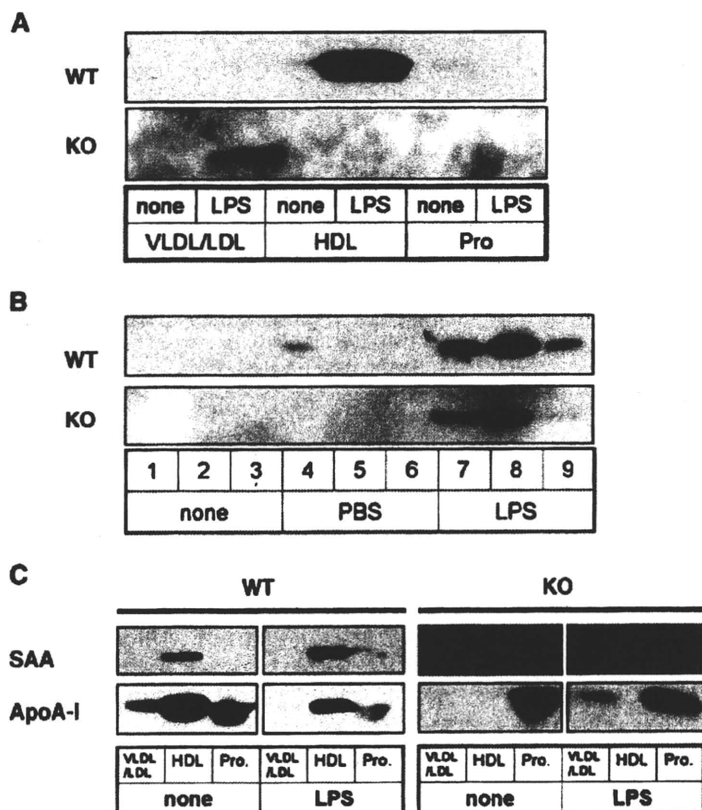

Fig. 2. Association of serum amyloid A (SAA) with lipoprotein fractions. A: Plasma SAA distribution in mice after LPS injection. Plasma was collected at 0 h (none) and 24 h (LPS) after LPS injection from WT and ABCA1-KO mice, and SAA was analyzed by Western blot analysis in VLDL/LDL, HDL, and protein (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.

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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[a]	1.22 ± 0.05[a]
	KO	0.10 ± 0.02	0.10 ± 0.03	0.25 ± 0.05
Phospholipid	WT	1.07 ± 0.05	2.78 ± 0.40[a]	2.10 ± 0.02[a]
	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).

[a] 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.

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.

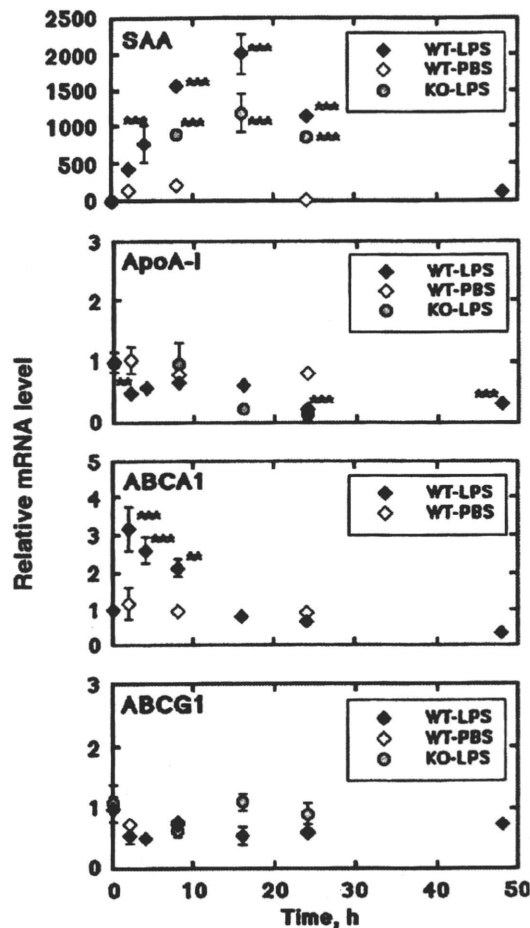

Fig. 3. Changes in the expression of SAA, apoA-I, ABCA1, and ABCG1 in the liver of ABCA1-KO (circles) and WT (diamonds) mice by LPS injection. Mice were treated with LPS (or with PBS), and the liver was collected at the times indicated (0, 2, 4, 8, 16, 24, and 48 h for WT mice and 0, 4, 8, 16, and 24 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: ** $P < 0.01$, *** $P < 0.001$.

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

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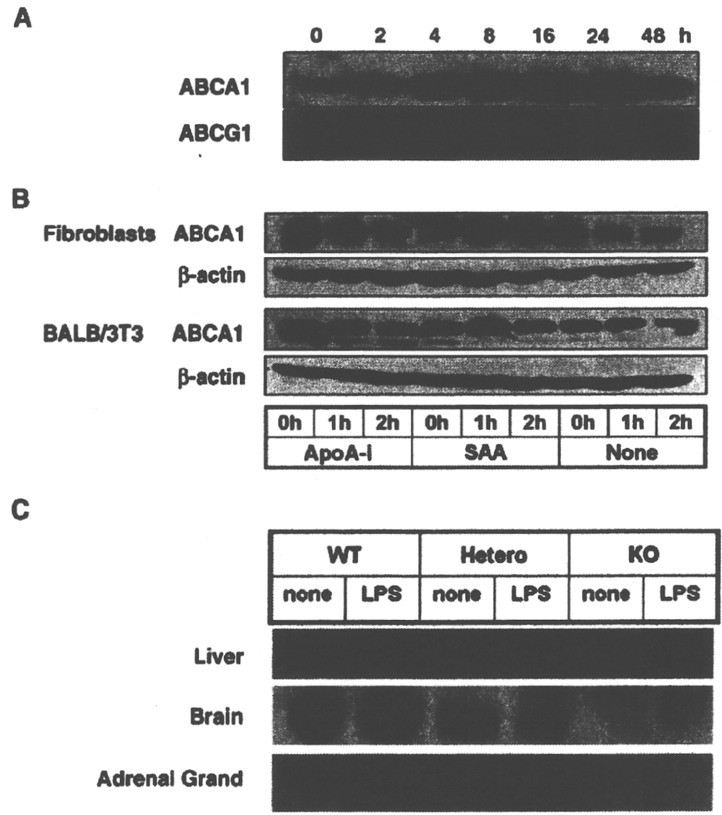

Fig. 4. A: Protein levels of liver ABCA1 and ABCG1 in LPS-treated WT mice at the indicated times. Each protein was analyzed by Western blotting (100 μg protein/lane). B: Retardation of the ABCA1 decay rate by lipid-free SAA and apoA-I. Fibroblasts prepared from WT mice and BALB/3T3 cells were incubated 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).

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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

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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.

This work was supported in part by grants-in-aid from the Ministry of Education, Science, Technology, Culture, and Sports of Japan, by operating grants from the Ministry of Health, Welfare, and Labor, and by the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation. This project was also supported in part by a Grant-in Aid for Research in Nagoya City University (S.A-D.) and by the Medical Science Promotion Fund of the 24th General Assembly of the Japanese Association of Medical Sciences (S.A-D.). The authors thank Dr. Takashi Miida of the Medical Laboratory Division of the University Medical and Dental Hospital, Niigata University, for his valuable suggestions. Technical support was provided by Kuniko Okumura-Noji and Mariko Hato for the preparation of human apoA-I.

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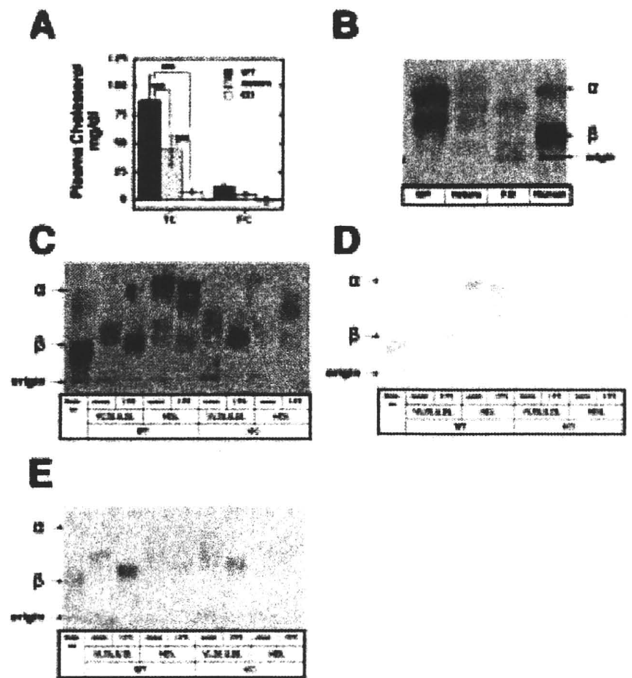
Supplementary Data 1. Effect of the LPS treatment on plasma HDL in mice. **(A)** Plasma lipoprotein lipid concentration at the baseline condition. Total cholesterol (TC) and free cholesterol (FC) of WT (gray columns), ABCA1-hetero (meshed columns) and ABCA1-KO mice (white columns) are shown. Each value 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 as 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 (J818 and J819, 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 LXR| mRNA in the liver after injection of LPS in WT mice. The liver was removed and LXR| 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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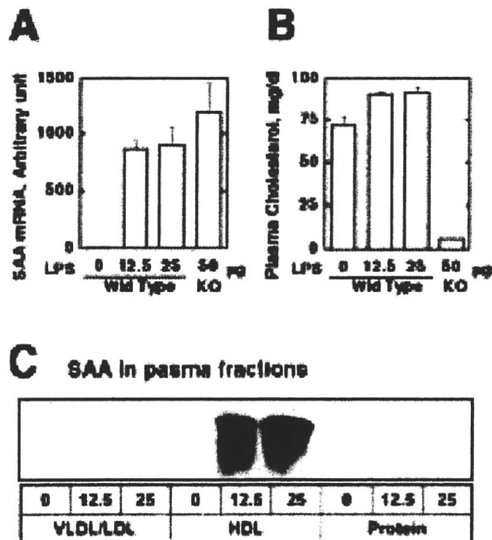

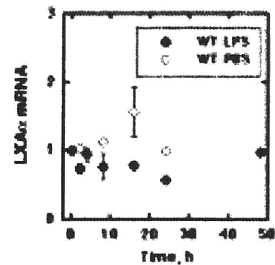

J. Biochem. **143**, 611–616 (2008)
doi:10.1093/jb/mvn008

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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Received October 30, 2007; accepted January 15, 2008; published online January 23, 2008

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^{TM} (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

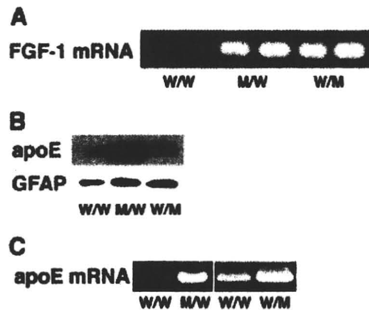

Fig. 1. **Expression of FGF-1 and apoE in astrocytes.** (A) Expression of FGF-1 mRNA in astrocytes prepared by 1-week primary and 1-week secondary culture (W/W), 1-month primary and 1-week secondary culture (M/W) and 1-week primary and 1-month secondary culture (W/M). Total cellular RNA was extracted 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

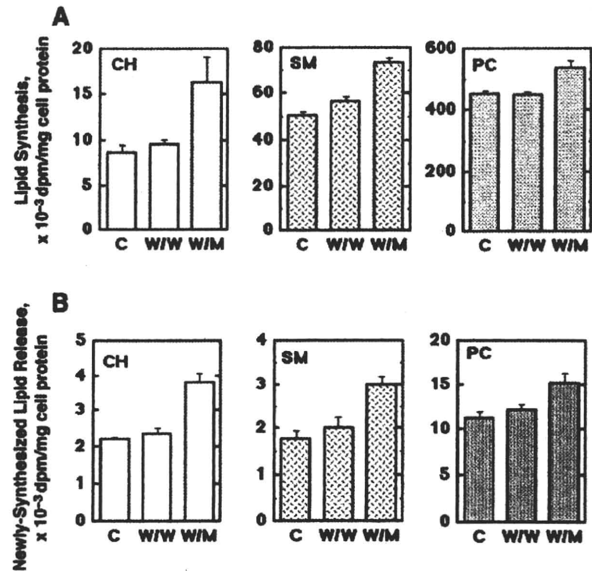

Fig. 2. **Effect of the conditioned medium of W/M cells on rat W/W astrocytes.** (A) Lipid biosynthesis was examined in W/W cells after stimulation by the conditioned medium of W/W and W/M cells. W/W cells were incubated in 500 μl of fresh 0.1% BSA/F-10 plus 500 μl of 0.1% BSA/F-10 or plus 500 μl of 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

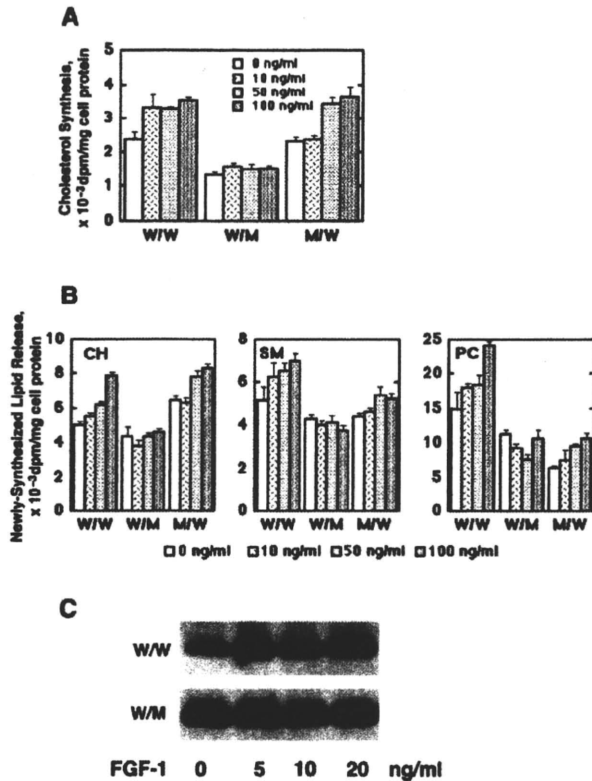

Fig. 3. **Effect of FGF-1 on astrocytes.** (A) W/W, W/M and M/W cells were incubated with FGF-1 (0, 10, 50 and 100 ng/ml) in 0.1% BSA/F-10 for 24 h. The cells were washed and labelled with 20 μCi/ml of [^{3}H]-acetate in a fresh 0.02% BSA/F-10 for 2 h. After washing, lipid was extracted from the cells 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.

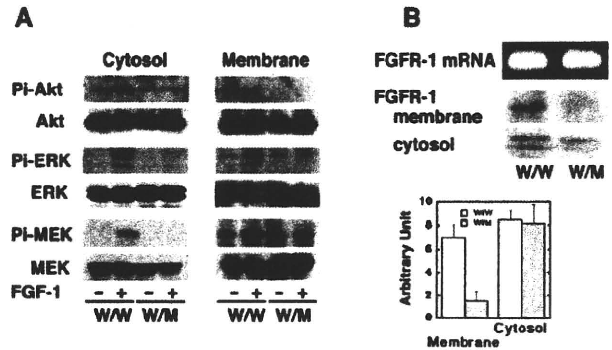

Fig. 4. **Analysis of the signalling pathways in rat astrocytes.** (A) Phosphorylation of signal-related proteins in W/W and W/M cells. The cells were washed and incubated in 0.1% BSA/F-10 for 16 h. After washing, the cells were treated with FGF-1 (50 ng/ml) for 5 min, and the cytosol and membrane fractions 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.

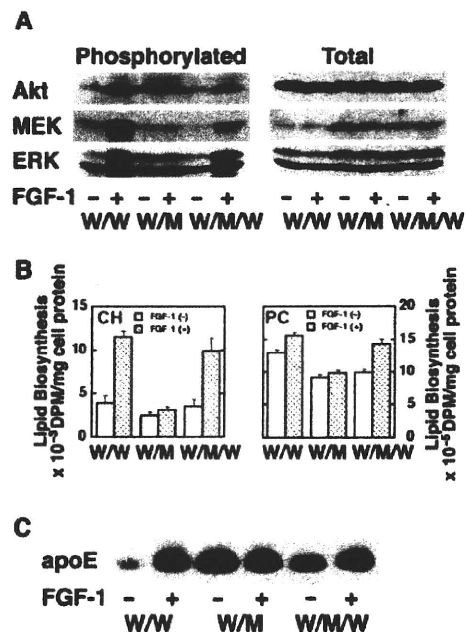

Fig. 5. **Recovery of reactivity of astrocytes to FGF-1 by short tertiary culture.** W/M cells were transferred to the tertiary culture of 1 week and analysed for the reactivity to FGF-1. The experiments were conducted in the same manner as those in Figs 3 and 4. (A) Phosphorylation of signalling proteins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

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 extra-cellular 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

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.

This work was supported by International HDL Award Program, in part by grants-in-aid from The Ministries of Education, Science, Technology, Culture and Sports, and of Health, Welfare and Labour of Japan, and by the Program for Promotion of Fundamental Studies in Health Sciences of National Institute of Biomedical Innovation.

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