Serum amyloid A generates high density lipoprotein with cellular lipid in an ABCA1- or ABCA7-dependent manner

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Abstract **Serum amyloid A (SAA) is an amphiphilic helical protein that is found associated with plasma HDL in various pathological conditions, such as acute or chronic inflammation. Cellular lipid release and generation of HDL by this protein were investigated, in comparison with the reactions by apolipoprotein A-I (apoA-I) and several types of cells that appear with various specific profiles of cholesterol and phospholipid release. SAA mediated cellular lipid release from these cells with the same profile as apoA-I. Upregulation of cellular ABCA1 protein by liver X receptor/retinoid X receptor agonists resulted in an increase of cellular lipid release by apoA-I and SAA. SAA reacted with the HEK293-derived clones that stably express human ABCA1 (293/2c) or ABCA7 (293/6c) to generate cholesterol-containing HDL in a similar manner to apoA-I. Dibutyryl cyclic AMP and phorbol 12-myristate 13-acetate, which differentiate apoA-I-mediated cellular lipid release between 293/2c and 293/6c, also exhibited the same differential effects on the SAA-mediated reactions. No evidence was found for the ABCA1/ABCA7-independent lipid release by SAA. Characterization of physicochemical properties of the HDL revealed that SAA-generated HDL particles had higher density, larger diameter, and slower electrophoretic mobility than those generated by apoA-I. These results demonstrate that SAA generates cholesterol-containing HDL directly with cellular lipid and that the reaction is mediated by ABCA1 and ABCA7.—**Abe-Dohmae, S., K. H. Kato, Y. Kumon, W. Hu, H. Ishigami, N. Iwamoto, M. Okazaki, C-A. Wu, M. Tsujita, K. Ueda, and S. Yokoyama. **Serum amyloid A generates high density lipoprotein with cellular lipid in an ABCA1- or ABCA7-dependent manner.** ***J. Lipid Res.*** **2006.** 47: **1542–1550.**

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Serum amyloid A (SAA) is a protein family that comprises acute-phase and constitutive members, both of which are synthesized mainly in the liver and the former is in reaction to the inflammatory status (1). Although constitutive SAA (e.g., SAA4 in human) is identified only in human and mouse, acute-phase SAA (e.g., SAA1 and SAA2 in human) is found in all of the vertebrates investigated and is highly conserved across evolutionarily distinct species (1). Acute-phase SAA is a major acute-phase reactant, showing up to 1,000-fold increase in human plasma during inflammation (2). Increase of this protein is also found with chronic inflammatory diseases. Acute-phase SAA is experimentally induced in mice by intraperitoneal injection of lipopolysaccharide (LPS), a potent endotoxin in septic shock (3). SAA has a multisegment structure of amphiphilic helix that is very similar to helical apolipoprotein (4) and is found associated with HDL, especially HDL_3, in the circulation (5).

SAA may play an antipathogenic role in inflammatory processes as it binds to outer membrane protein A of Gram-negative bacteria (6). However, it could also be pro-inflammatory. LPS binds the scavenger receptor class B type I (SR-BI) and is internalized to stimulate cells to produce cytokines such as tumor necrosis factor-α (TNF-α), interleukin(IL)-1α, IL-1β, IL-6, and IL-8, IL-1ra, and soluble tumor necrosis factor receptor-II (TNFR-II) (7–9), and SAA itself may also contribute to the stimulation of cells via SR-BI to produce cytokines such as IL-1β, IL-8, IL-1ra, and soluble TNFR-II but not TNFα or IL-6, although

Abbreviations: apoA-I, apolipoprotein A-I; dBcAMP, dibutyryl cyclic AMP; DF medium, 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium; D-PBS, Dulbecco's phosphate-buffered saline; IL, interleukin; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; SAA, serum amyloid A; SR-BI, scavenger receptor class B type I; TNF-α, tumor necrosis factor-α; TNFR-II, tumor necrosis factor receptor-II.

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to a lesser extent than LPS (7, 10). In addition, SAA is a precursor of amyloid A protein, the major component of amyloid fibrils in secondary amyloidosis (11, 12).

A more complicated argument is that SAA may modulate the effect of LPS through HDL metabolism. The effect of LPS is blocked by physiological ligands of SR-BI, HDL, apolipoprotein A-I (apoA-I), and amphiphilic α-helical peptides (9). As the HDL containing SAA competes with normal HDL for SR-BI binding (10, 13), an increase of such HDL may interfere with the effect of LPS. Infusion of HDL protected mice from the LPS-induced endotoxin shock status (14), which seems to be consistent with the view that HDL traps LPS from direct interaction with inflammatory cells in the interstitial space (15, 16). HDL particles containing SAA are different from regular apoA-I-containing HDLs in their physicochemical properties (3, 5), and they may result in different biological functions (1). Displacement of apoA-I from HDL by SAA causes an enhancement of HDL clearance and thereby a reduction of HDL (5, 17). A recent report, however, indicated that SAA, whether free or lipid-bound, could compete with HDL binding to SR-BI and interfere with cellular cholesteryl ester uptake from HDL (10, 13). Thus, reports of the roles of SAA in the pathogenesis of inflammation and HDL metabolism are controversial and unclear.

HDL plays a central role in cholesterol transport from extrahepatic peripheral cells to the liver as a major pathway for cholesterol catabolism. This pathway is also thought important to act against atherogenesis, as intracellularly accumulated cholesterol in the vascular wall can be removed by this mechanism. One of the rate-limiting steps of this pathway is the release of cellular cholesterol to HDL, and two independent mechanisms have been identified for this step (18): nonspecific cholesterol exchange between cell membrane and extracellular lipoproteins, and assembly of new HDL particles with cellular lipid and lipid-free helical apolipoproteins. The latter seems a major source of plasma HDL, as the cells from patients with familial HDL deficiency (Tangier disease) lack this reaction (19, 20). ABCA1 was identified as essential for HDL biogenesis (21–25). ABCA7 was also shown to mediate HDL biogenesis when expressed in HEK293 and L929 cells (26, 27).

Recently, SAA was reported to induce cellular lipid release both in ABCA1-dependent and -independent manners (28). Here, we have characterized the SAA-cell interaction in the context of HDL biogenesis using a series of cell lines. It was demonstrated that SAA is an analog of helical apolipoprotein and is fully capable of generating new HDL particles with cellular lipid in the presence of ABCA1 or ABCA7 in the cell membrane. HDLs generated with SAA appeared to have different physicochemical properties than those generated with apoA-I.

MATERIALS AND METHODS

Materials

ApoA-I was isolated from a human plasma HDL fraction (density, 1.09–1.21) and stored at −80°C until use as described previously (29). A stock solution (1 mg/ml) was prepared and stored at 4°C as described previously (30). SAA used in this study was recombinant human SAA corresponding to human SAA1α except for three amino acids (catalog number 300-13; PeproTech EC, London, UK), and a stock solution (1 mg/ml) was prepared according to the manufacturer's instructions and stored at 4°C. ApoA-I protein was undetectable in the SAA stock solution by Coomassie Brilliant Blue staining or Western blotting (data not shown). The sources of the other reagents were as follows: phorbol 12-myristate 13-acetate (PMA) and 9-*cis* retinoic acid were from Wako (Osaka, Japan); liver X receptor agonist TO901317 was from Sigma; dibutyryl cyclic AMP (dBcAMP) was from Seikagaku Corp. (Tokyo, Japan); and protein kinase C inhibitor Gö6976 was from Biomol Research Laboratories.

Cell culture

THP-1, HEK293, and L929 cells were obtained from the Health Science Research Resources Bank. HEK293 clones of 293/2c and 293/6c were established in our laboratory; they stably express human ABCA1-green fluorescent protein and ABCA7-green fluorescent protein, respectively (27). CHO-K1 cells, which produce cholesterol-containing HDL were from the American Type Culture Collection. The cells were maintained in medium supplemented with 10% (v/v) fetal calf serum under a humidified atmosphere of 5% CO_2 and 95% air at 37°C. RPMI 1640 medium was used for THP-1 cells, and a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF medium) was used for all other cells. For differentiation of THP-1 cells, cells were subcultured in six-well trays (Techno Plastic Products AG, Trasadingen, Switzerland, catalog number 92406) at a density of 3.0×10^6 cells/well in the presence of 320 nM PMA for 72 h (31).

Cellular lipid release assay

Cells were subcultured in six-well trays at the indicated density with 10% fetal calf serum-containing medium. After 48 h of incubation, the cells were washed once with Dulbecco's phosphate-buffered saline (D-PBS), except that buffer H [Hank's balanced salt solution containing 20 mM HEPES-KOH (pH 7.5) and 14 mM glucose] was used for HEK293, 293/2c, and 293/6c (27), and incubated in 1 ml/well DF medium containing 0.02% BSA (0.02% BSA/DF medium) and the compounds indicated. The PMA-treated THP-1 cells were further washed with D-PBS and incubated with apoA-I or SAA. The PMA-untreated THP-1 cells were directly subcultured in six-well trays at a density of 3.0×10^6 cells/well and cultured in 0.02% BSA/DF medium containing apoA-I or SAA. Lipid content in the medium and cells was determined after 24 h of incubation. Procedures for lipid extraction and enzymatic assays for cholesterol, cholesteryl ester, and choline-phospholipids were described previously (30).

Lecithin:cholesterol acyltransferase and lipase activities of the culture medium were determined using LCAT kit-S from Alfresa Pharma Corp. (catalog number 4987274862199; Tokyo, Japan) and an LIP Roche/Hitachi from Roche Diagnostics K.K. (catalog number 11821791 216; Tokyo, Japan), respectively.

Western blotting

A rabbit anti-ABCA1 antiserum (32, 33), a rat monoclonal anti-ABCA7 antibody, KM3095 (34), and a rat monoclonal anti-SAA antibody (Cell Sciences) were used to detect the indicated proteins.

Lipoprotein analysis

ABCA1- or ABCA7-transfected cells, 293/2c and 293/6c, were subcultured in 100 mm dishes at a density of 6.0×10^6 cells/dish,

cultured as described above, and stimulated with 5 ml/dish 0.02% BSA/DF medium containing 10 μg/ml SAA for 24 h. The conditioned medium was analyzed by sucrose density gradient ultracentrifugation as described previously (27, 35). It was also analyzed by an HPLC system with two tandem gel permeation columns (Lipopropak XL, 7.5 mm × 300 mm; Tosoh) at a flow rate of 700 μl/min and two parallel online enzymatic lipid detection systems (350 μl/min each) (36, 37) to evaluate the size distribution of the lipoprotein particles (38, 39) (Skylight Biotech, Inc., Akita, Japan). The system was calibrated by latex beads and high-molecular-weight standards for apparent spherical diameters of the subjects (36, 37).

For electron microscopy, the HDL fraction was isolated by ultracentrifugation from the conditioned medium at a density of 1.21 g/ml adjusted with NaBr or sucrose at 90,000 rpm in a Hitachi CS100 ultracentrifuge with an S100AT6 rotor (4.8×10^5 *g*) for 16 h (NaBr) or 48 h (sucrose) at 4°C. The top fraction (300 μl) of each tube (3 ml) was collected as HDL. The HDL fractions from eight tubes of the sucrose solution were pooled and further concentrated by centrifugation for another 48 h at 1.21 g/ml with sucrose. HDL samples were dialyzed against 10 mM ammonium bicarbonate, pH 7.9, at 4°C overnight. They were negatively stained with 0.5% uranium acetate and examined with a Hitachi 7100 electron microscope as described (40, 41). Long diameters (major axes) of the HDL particles were measured graphically using NIH Image 1.63 software.

To evaluate the electrophoretic mobility of lipoproteins, the conditioned medium was analyzed using an agarose gel electrophoresis system (Paragon System; Beckman) after concentration by an ultrafiltration filter (Amicon Ultra-15; Millipore).

Statistical analysis

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

RESULTS

Cellular lipid was released by SAA and apoA-I from THP-1 cells (**Fig. 1**). From the undifferentiated cells, cholinephospholipid was a major component, suggesting that the HDL generated was cholesterol-poor. When the cells were differentiated with PMA, the lipid release was increased but more prominently in cholesterol, indicating the generation of cholesterol-rich HDL. The lipid-release profile was also examined for CHO-K1, L929, and HEK293 cells (**Fig. 2**). These cell lines represent cells that release both phospholipid and cholesterol, phospholipid only, and no lipid, respectively, by apoA-I, as we reported in previous works (27, 42). The results with SAA were the same as those with apoA-I.

Transcriptional activation of the ABCA1 gene resulted in an increase of the SAA-mediated lipid release. A retinoid X receptor ligand, 9-*cis* retinoic acid, and a liver X receptor ligand, TO901317, increased ABCA1 synergistically in CHO-K1 cells (**Fig. 3A**). In these conditions, the lipid release mediated by apoA-I and by SAA both increased in parallel (Fig. 3B, C). That apoA-I increased ABCA1 is consistent with our previous reports that ABCA1 is stabilized against calpain by helical apolipoprotein (33, 43, 44), and SAA showed the same effect (Fig. 3A).

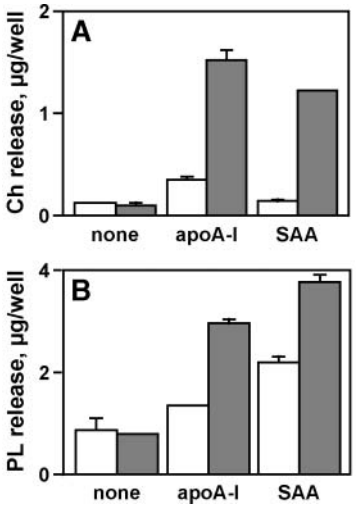

Fig. 1. Release of cholesterol (Ch; A) and choline-phospholipids (PL; B) from THP-1 cells. Undifferentiated (open columns) and differentiated (shaded columns) THP-1 cells were prepared as described in the text. Cells were washed once with Dulbecco's phosphate-buffered saline (D-PBS) and incubated with 0.02% BSA/DF medium (0.02% BSA and a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium) containing nothing (none), 10 μg/ml apolipoprotein A-I (apoA-I), or 10 μg/ml serum amyloid A (SAA) for 24 h. Cholesterol and choline-phospholipid in the medium were measured as described in the text. Data shown represent averages ± SD of duplicate measurements in one of three independent experiments yielding similar results. Error bars are not shown when they lie within the columns.

Figure 4 shows dose-dependent profiles of lipid release by apoA-I and SAA from cells to which ABCA1 or ABCA7 was transfected (293/2c or 293/6c, respectively). Although the ABCA1-mediated lipid release was nearly similar between apoA-I and SAA (Fig. 4A, C), the SAA-mediated lipid release was substantially less efficient with ABCA7 (Fig. 4B, D). dBcAMP and PMA had differential effects on the apoA-I-mediated cellular lipid release between ABCA1- and ABCA7-transfected cells, perhaps at posttranscriptional steps (27). These compounds showed very similar differential effects on SAA-mediated lipid release between the clones (**Fig. 5**). The SAA-mediated release of cholesterol and phospholipid from the ABCA1-transfected cells was increased by dBcAMP and PMA, and the effect of PMA was canceled by an inhibitor of protein kinase C, Gö6976 (Fig. 5A, C). In contrast, SAA-mediated lipid release from the ABCA7-transfected cells was not influenced by dBcAMP. PMA suppressed the ABCA7-dependent lipid release mediated by SAA, and Gö6976 canceled the effect (Fig. 5B, D). All of these results were consistent with our previous findings with apoA-I (27).

HDL generated by SAA was analyzed by density gradient ultracentrifugation (**Fig. 6A**). Cholesterol and phospholipid released from 293/2c were both recovered in the fractions with a density peak at ~1.10 g/ml, and those from 293/6c were recovered in the fractions with a density peak at 1.14 g/ml. SAA protein was detected in the lipid-containing fractions (data not shown). SAA used in this

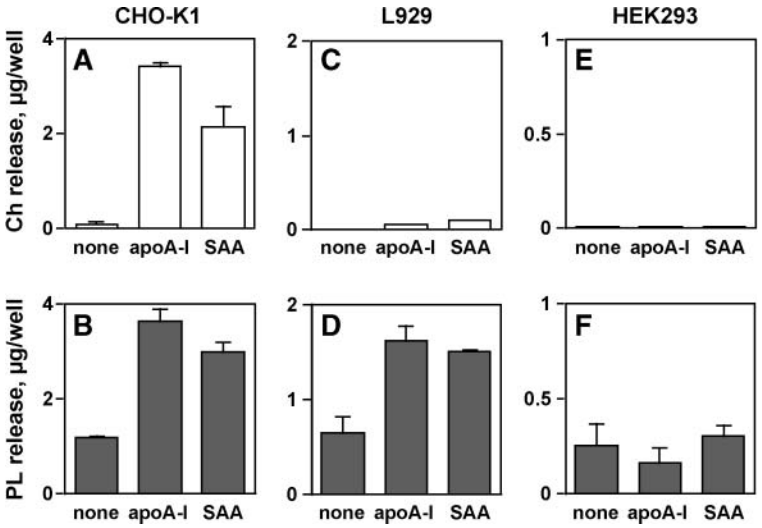

Fig. 2. Release of cholesterol (Ch; A, C, E) and choline-phospholipids (PL; B, D, F) from CHO-K1 (A, B), L929 (C, D), and HEK293 (E, F) cells. CHO-K1, L929, and HEK293 cells were subcultured in six-well trays at densities of 5.0×10^5, 5.0×10^5, and 1.0×10^6 cells/well, respectively. After 48 h of incubation, cells were washed once and treated as described for Fig. 1. Data represent averages ± SD of duplicate measurements in one of two independent experiments yielding similar results.

study was recombinant protein and had no detectable apoA-I (see Materials and Methods), so that SAA generated cholesterol-containing HDL from the cells in the presence of ABCA1 or ABCA7.

The size distribution of the HDL particles was analyzed by HPLC. The apoA-I-conditioned medium showed two peaks, consistent with our previous findings (39). Large cholesterol-rich particles are predominant in the HDL generated with the ABCA1-transfected 293/2c cells, whereas small cholesterol-poor particles are dominant with the ABCA7-transfected 293/6c cells (Fig. 6B). In contrast, the peaks found in the same analysis of the SAA-conditioned medium appeared large, single, broad, and rather symmetric (Fig. 6C).

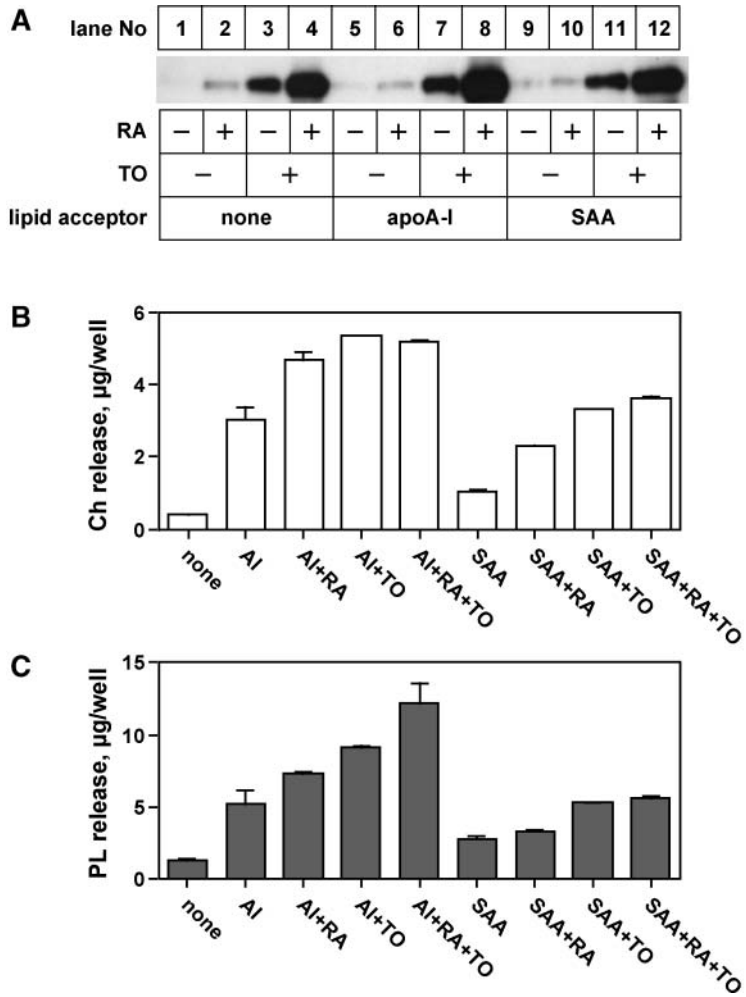

Fig. 3. Effects of 9-*cis* retinoic acid and TO901317 on cellular ABCA1 protein level and lipid release by apoA-I and SAA. CHO-K1 cells were subcultured in six-well trays at a density of 5.0×10^5 cells/well. After 48 h of incubation, cells were washed with D-PBS, and then 0.02% BSA/DF medium containing the compounds indicated was added. The final concentrations of 9-*cis* retinoic acid (RA), TO901317 (TO), apoA-I (AI), and SAA were 5 µg/ml, 10 µM, 10 µg/ml, and 10 µg/ml, respectively. Cells were cultured for another 24 h, and the cells and medium were collected for lipid measurement. The membrane fraction was prepared from the remaining cells for Western blot analysis of ABCA1. A: ABCA1 analysis by Western blotting. Membrane protein (100 µg/lane) was analyzed as described in the text. B, C: Release of cholesterol (Ch) and choline-phospholipid (PL), respectively, into the medium. Results shown are averages ± SD for two samples.

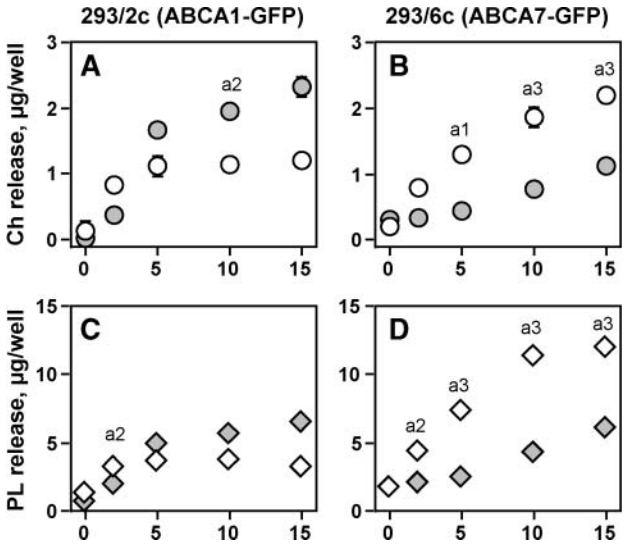

Fig. 4. Release of cholesterol (Ch; A, B) and choline-phospholipid (PL; C, D) from 293/2c (A, C) and 293/6c (B, D) cells. Cells were subcultured in six-well trays at a density of 1.0×10^6 cells/well and cultured for 48 h. After washing with buffer H, cells were incubated in 1 ml/well 0.02% BSA/DF medium containing apoA-I (open symbols) or SAA (shaded symbols) at the concentrations indicated. Cholesterol and choline-phospholipid were determined in the medium after 24 h of incubation. Results represent averages ± SD of duplicate assays in one of three independent experiments yielding similar results. Error bars are not shown when they lie within the symbols. Statistical significance for differences is indicated as a1, a2, and a3, for $P < 0.05$, 0.01, and 0.001 between apoA-I and SAA at the concentrations matched. GFP, green fluorescent protein.

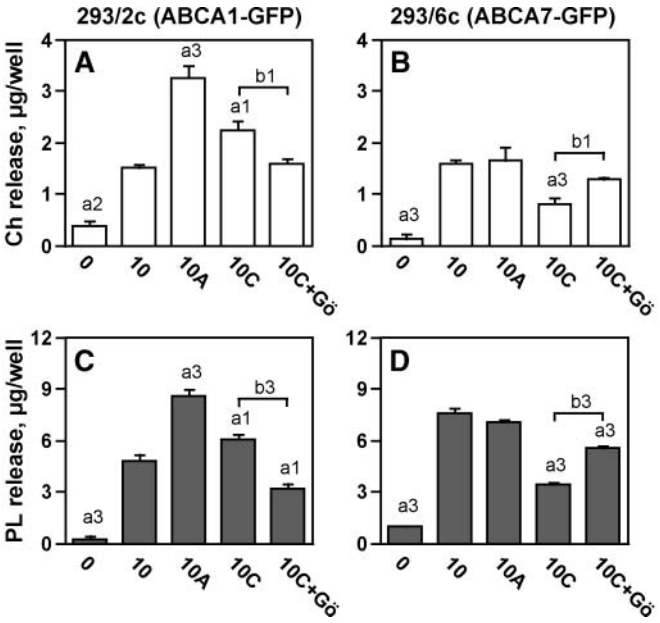

Fig. 5. Effects of dibutyryl cyclic AMP (dBcAMP), phorbol 12-myristate 13-acetate (PMA), and Gö6976 on SAA-mediated lipid release from 293/2c (A, C) and 293/6c (B, D) cells. Cells were cultured for 48 h as described for Fig. 4. The cells were then washed with buffer H and cultured in 0.02% BSA/DF medium with 10 μg/ml SAA (10) and the indicated concentration of compounds (dBcAMP, 10A; PMA, 10C; and PMA plus Gö6976, 10C+Gö) or in 0.02% BSA/DF alone (0). Lipids in the medium were determined after 24 h of incubation. Results represent averages ± SD of two or three samples in one of three independent experiments that yielded similar results. Error bars are not shown when they lie within the symbols. Statistical significance for differences is indicated as a1, a2, and a3, for $P < 0.05$, 0.01, and 0.001 against the data for 10 μg/ml SAA and as b1 and b3 for $P < 0.05$ and 0.001 between the data indicated. Ch, cholesterol; GFP, green fluorescent protein; PL, choline-phospholipid.

Negative staining electron microscopy revealed that the apoA-I-generated HDL consisted of predominantly "disc-like" particles that tend to aggregate into a rouleau formation (**Fig. 7A, C**). The SAA-generated HDL apparently contained fewer disc-like particles and had less tendency to aggregate (Fig. 7E, G). The rouleau formation was less obvious when HDL particles were prepared with sucrose (data not shown). The distribution of particle size was obtained by measuring the major axis of the particles graphically (Fig. 7B, D, F, H). The diameter peak of the apoA-I-generated HDL was larger with 293/2c (ABCA1) than with 293/6c (ABCA7). The SAA-generated HDL was larger than the apoA-I-generated HDL, consistent with the results of HPLC.

Sizes and lipid compositions of the HDL generated from 293/2c and 293/6c are listed in **Table 1**. The results obtained by two independent procedures, HPLC and ultracentrifugation, were in good agreement. Stokes diameters of the particles in HPLC were substantially larger than their major axes measured in the electron micrographs as a result of their disc-like amorphous and asymmetric shape. Almost all of the lipids were unesterified cholesterol and phospholipid, and the cholesterol-to-phospholipid ratio was less in the ABCA7-generated HDL (293/6c) than in the ABCA1-mediated HDL (293/2c). The contents of cholesteryl ester and triglyceride were both negligible in the HDLs. The level of cholesteryl ester was also negligible in the apoA-I-conditioned medium of all cells, including THP-1 and CHO-K1 (data not shown). Neither lecithin:cholesterol acyltransferase nor lipase activity was detected in the conditioned medium of 293/2c and 293/6c as well as parent HEK293 cells with or without lipid acceptors (data not shown), indicating that the differences in lipid composition or particle diameter are not caused by secondary modification of the HDL.

The electrophoretic mobility of lipoproteins generated in the SAA-conditioned medium was the same between 293/2c and 293/6c cells and slower than that generated in the apoA-I-conditioned medium (**Fig. 8**).

DISCUSSION

The results are summarized as follows. *1*) SAA interacts with the cells and induces cellular lipid release in a very similar manner to apoA-I, with respect to the phospholipid and cholesterol release profiles. *2*) Also like the apoA-I-cell interaction, the reaction requires ABCA1 or ABCA7, except that the reaction of SAA is less effective than that of apoA-I with ABCA7 in HEK293 cells. *3*) HDL generated

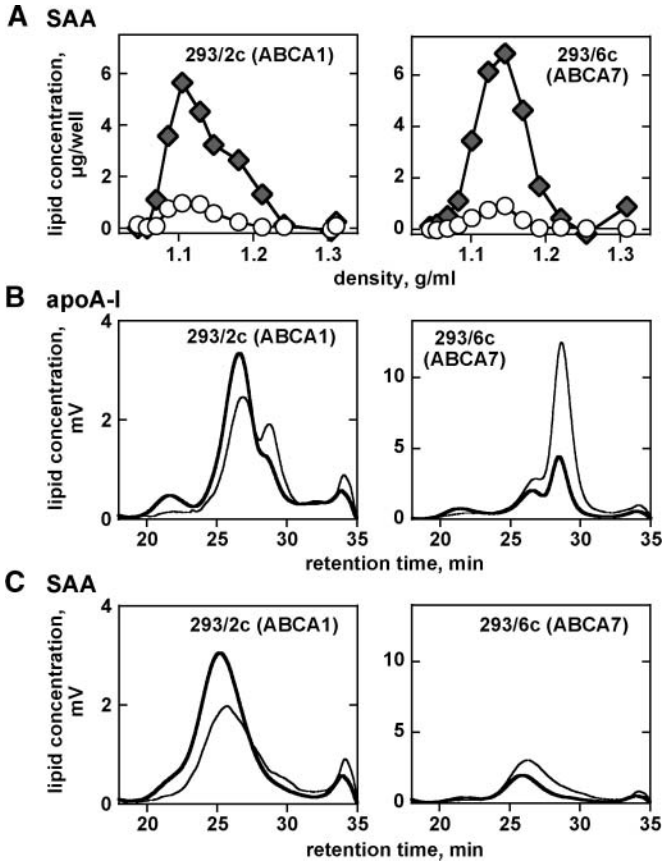

Fig. 6. Characterization of the SAA-derived HDL particles. A: Density gradient analysis of the SAA-conditioned medium of 293/2c and 293/6c cells (ABCA1 and ABCA7). Cells were cultured and stimulated with 10 μg/ml SAA. The medium was analyzed by density gradient ultracentrifugation as described in Materials and Methods. Cholesterol (open circles) and choline-phospholipid (shaded diamonds) were measured for each fraction. B, C: Size exclusion HPLC analysis of the conditioned medium of 293/2c and 293/6c cells (ABCA1 and ABCA7). Cells were cultured for 48 h as described for Fig. 4, washed with buffer H, and incubated in 0.02% BSA/DF medium with 10 μg/ml apoA-I (B) or SAA (C) for 24 h. The medium was analyzed by HPLC as described in the text, and cholesterol (thick lines) and choline-phospholipid (thin lines) were monitored online in the eluent with absorbance at 550 and 585 nm, respectively, and are expressed in mV calibrated as 30.0 nM/mV for cholesterol and 55.6 nM/mV for choline-phospholipid.

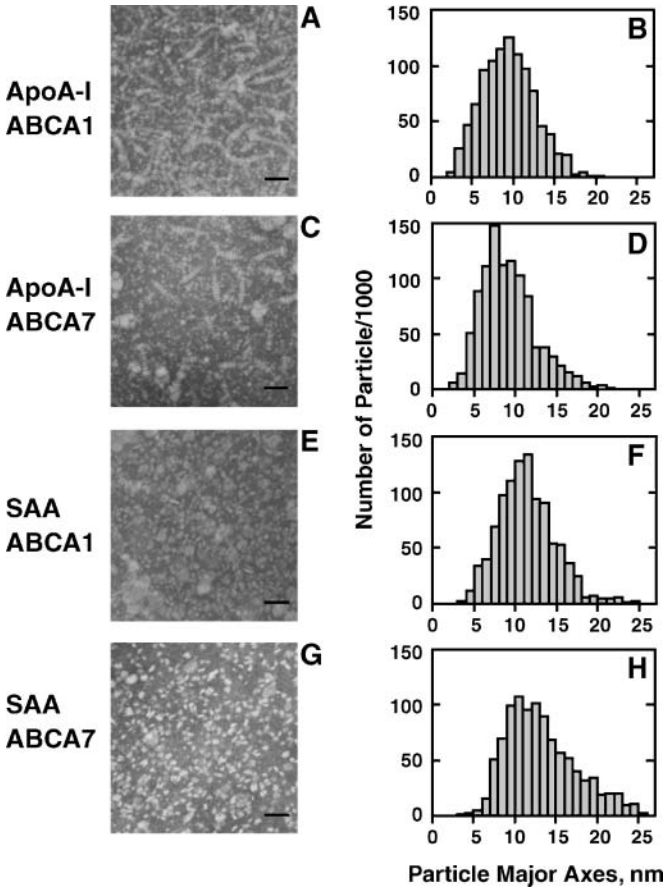

Fig. 7. Electron micrographs of negatively stained HDL particles from 293/2c and 293/6c cells. Conditioned medium was prepared as described for Fig. 6, and HDL fractions were prepared with NaBr as described in Materials and Methods. Each scale bar represents 50 nm in panels A, C, E, G. Histograms B, D, F, H represent the size distribution of HDL particles in panels A, C, E, G, respectively, based on graphic measurement of the major axes of particles using NIH Image 1.63 software.

by SAA is substantially different from that produced by apoA-I in its physicochemical properties.

HDL is generated by the ABCA1-mediated reaction with various peptides having amphiphilic α-helical segments, including helical apolipoproteins (35, 45), synthetic peptides (46, 47), and even those containing only D-amino acids (44, 48). Thus, there is no specific requirement of amino acid sequence for the biogenesis of HDL except for amphiphilic helices as a key conformation. A prediction from the secondary structure of human SAA1 indicated two amphiphilic α-helix regions (4), fulfilling this requirement. Amphiphilic segment conformation is also related to the stabilization of ABCA1 from calpain-mediated degradation (33), perhaps related to the phosphorylation of ABCA1 (43, 44). ABCA1 apparently increased upon incubation with SAA, so this protein also seems capable of inducing this effect.

SAA reproduced a profile of the apoA-I-mediated reaction. Protein kinase C activation increased the ABCA1-mediated lipid release but suppressed that mediated by ABCA7. The lipid composition of the HDL exhibited a similar difference between ABCA1 and ABCA7 by both apoA-I and SAA (27, 39). The cellular profile of apoA-I to produce cholesterol-rich HDL, cholesterol-poor HDL, and no HDL (42) is also reproduced by SAA. However, SAA induces as much lipid release as apoA-I from the ABCA1-transfected cells (293/2c) but significantly less than apoA-I from the ABCA7-transfected cells (293/6c).

It was reported recently that SAA removes cellular lipids in an ABCA1-dependent manner but also induces substantial lipid release even without ABCA1, in contrast to apoA-I (28). This is inconsistent with our present results that there was no significant lipid release observed with HEK293 cells without transfection of ABCA1 or ABCA7. The reason for this disagreement is unknown. The mass of cholesterol and choline-phospholipids in the medium

TABLE 1. Apparent size and lipid composition of the HDLs generated by apoA-I and SAA from HEK293 cells in which ABCA1 or ABCA7 was transfected and expressed (293/2c or 293/6c)

Cell	Protein	HDL size: Stoke's diameter, Ch/PL peak 1	HDL size: Stoke's diameter, Ch/PL peak 2	HDL size: Electron micrograph, peak range	Ch to PL in HDL: HPLC	Ch to PL in HDL: Ultracentrifuge
		nm			*w/w*	
293/2c	ApoA-I	15.2/14.3	10.2/9.8	9–10	0.258	0.276
	SAA	20.2/18.2		11–12	0.254	0.305
293/6c	ApoA-I	15.3/14.4	10.4/10.0	7–8	0.100	0.137
	SAA	17.5/16.2		10–11	0.132	0.181

ApoA-I, apolipoprotein A-I; Ch, cholesterol; PL, choline-phospholipid; SAA, serum amyloid A. For the HPLC analysis, the size represents Stokes diameters of the peaks of cholesterol and choline-phospholipid based on column calibration by spherical standards (see text), and the lipid composition data were calculated by integration of the HDL peaks (see Fig. 6B, C). Peak 1 is predominant in the 293/2c-conditioned medium, and peak 2 is predominant in the 293/6c-conditioned medium when apoA-I is present (see Fig. 6B, C). For the ultracentrifugation analysis, the size represents the peak range of the histogram obtained by direct measurement of the major axes of the particles in the electron micrograph in Fig. 7, and the lipid composition data were obtained directly by measuring lipid in the HDL fraction isolated by ultracentrifugation in sucrose (see text). Lipids other than unesterified cholesterol and choline-phospholipid, such as cholesteryl ester and triglyceride, were negligible in both analyses. Data are representative of one of two independent experiments that yielded similar results.

was measured directly in our experiments, whereas in the previous report the lipid release by SAA was measured by counting radioactivity released into the medium after labeling the cell lipids and was expressed as a percentage of the count in cellular lipids. L929 cells, which express substantial levels of ABCA1 protein and release only phospholipid by apoA-I (27, 42), responded to SAA by releasing only phospholipid and no cholesterol (Fig. 2C, D), so it is likely that SAA does not induce significant ABCA1-independent lipid release. Cholesterol release from parent HEK293 and L929 cells was observed only when incubated with (2-hydroxypropyl)-β-cyclodextrin, an acceptor for diffusion-mediated cholesterol release (data not shown). Thus, we conclude that SAA induces cellular lipid release and generates HDL, dependent on ABCA1 or ABCA7.

The physicochemical properties of HDL produced by SAA were characterized in comparison with those generated by apoA-I. *1*) Sucrose density gradient ultracentrifugation data showed that the density peaks of SAA-generated HDL were higher (1.10 and 1.14 g/ml in 293/2c and 293/6c cells, respectively) than those obtained from apoA-I-generated HDL [1.08 g/ml (27)]. *2*) HPLC analysis demonstrated that HDL particles generated by SAA appeared as a single broad symmetric peak of relatively large size, whereas the apoA-I-generated HDL appeared in two distinct peaks, both of which were smaller than the SAA-generated HDL (39). *3*) Negative staining electron microscopy confirmed that the size distribution profiles of HDL were consistent with the results of HPLC and showed that rouleau formation was more obvious in the apoA-I-generated HDL than in the SAA-generated HDL. *4*) Agarose gel electrophoresis showed distinct differences in the surface charge of these particles, reflecting the isoelectric point values of apoA-I and SAA [5.40 and 6.35, respectively, calculated by the EMBL WWW Gateway to Isoelectric Point Service (http://www.embl-heidelberg.de/cgi/pi-wrapper.pl)]. These results are consistent with the finding that SAA-containing HDL particles isolated from patients with severe inflammatory disease (5) and from mice after intraperitoneal injection of LPS (3) are denser and larger than those from their normal counterparts. It should be noted that all of these physicochemical properties, including rouleau formation, are more dependent on the type of apolipoprotein than on contributing ABC transporters or the lipid composition of the generated HDL.

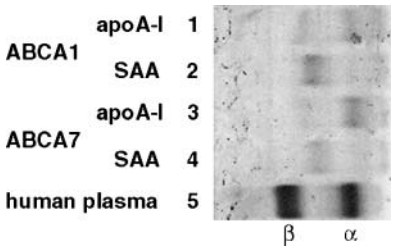

Fig. 8. Agarose gel electrophoresis of apoA-I- and SAA-containing HDL. 293/2c (ABCA1; lanes 1, 2) and 293/6c (ABCA7; lanes 3, 4) cells were incubated with 10 μg/ml apoA-I (lanes 1, 3) or SAA (lanes 2, 4) for 24 h as indicated for Fig. 6. Each conditioned medium was briefly centrifuged to remove cell debris and then processed by ultrafiltration. Medium concentrated by 20-fold was analyzed by agarose gel electrophoresis and Sudan Black staining. α and β indicate HDL and LDL, respectively, in human plasma.

SAA-containing HDL particles can also be produced by displacement of apoA-I by SAA. In vitro experiments in fact demonstrated that lipid-free SAA displaced preexisting apoA-I on HDL (5, 49). It is not known whether SAA-HDL produced by apoA-I displacement has the same properties as the SAA-generated HDL or what proportion of SAA-HDL is produced by the apoA-I displacement.

The presence of HDLs with different physicochemical properties may influence HDL metabolism. However, information from the literature is diverse. Four possible SAA receptors have been reported: FPRL1 (a G protein-coupled chemotactic receptor) (50); a receptor for advanced glycation end product (51); Tanis (a membrane selenoprotein) (52, 53); and SR-BI (10, 13). Among these,

SR-BI binds both lipid-free SAA and SAA-containing HDL and also binds apoA-I and apoA-I-containing HDL (13). SAA molecules were cointernalized with transferrin (10) in an SR-BI-dependent manner (13). SAA binding to SR-BI-expressing cells caused activation of the ERK1/2 and p38 pathway (10), which was proposed to be a mechanism for SAA-mediated cytokine production (7). These reactions were blocked by HDL, apoA-I, and amphiphilic α-helical peptides (10). These findings, however, do not clearly help us to understand the roles of SAA in HDL metabolism.

Thus, the functions of SAA and SAA-HDL are still to be determined. They may be partly responsible for host defense mechanisms. They may help to remove cholesterol from an inflammation site. Finally, they may just be a secondary protein to the inflammatory event. Studying the production mechanism of SAA-containing HDL should help us to understand the functions of SAA.

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