Recombinant human serum amyloid A ($apoSAA_p$) binds cholesterol and modulates cholesterol flux

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Abstract During acute inflammation, the serum amyloid A (apoSAA) proteins $apoSAA_1$ and $apoSAA_2$ are transiently associated with high density lipoproteins (HDL) in concentrations of as much as 1000-fold more than their concentrations during homeostasis; however, their effect on HDL function is unclear. Recombinant $apoSAA_p$, a hybrid of the closely related human $apoSAA_1$ and $apoSAA_2$ isoforms, was found to exhibit a high affinity for cholesterol. The adsorption of $apoSAA_p$ to polystyrene microtiter wells at physiological pH, temperature, and salt concentration was inhibited and reversed by cholesterol. $ApoSAA_p$, to a greater extent than apoA-I, albumin, or fetal bovine serum, enhanced diffusion of cholesterol from HDL across a membrane that retained molecules >3.5 kDa. Cholesterol from 25 nM to 125 μM inhibited binding of [^{3}H]cholesterol to 167 nM $apoSAA_p$. A cholesterol binding assay was developed to determine the dissociation constant for binding of [^{3}H]cholesterol to $apoSAA_p$; $K_d = 1.7 \pm 0.3 \times 10^{-7}$ M and the maximum binding capacity (B_{max}) is 1.1 ± 0.1 mol/mol. After binding cholesterol, the apparent size of $apoSAA_p$ as determined by gel filtration on Sephacryl S-100 was increased from 12 to 23 kDa. $ApoSAA_p$ enhanced free [^{14}C]cholesterol uptake from tissue culture medium by HepG2 cells, an effect that was dose dependent and blocked by polyclonal antibodies to human $apoSAA_1$ and $apoSAA_2$. $ApoSAA_p$, unlike apoA-I, was taken up from serum-free medium by HepG2 cells and appeared to be degraded by cell-associated enzymes. Unlike peritoneal exudate cells, human HepG2 hepatoma cells do not secrete an enzyme that degrades $apoSAA_p$. These results suggest that apoSAA can potentially serve as a transient cholesterol-binding protein. **—Liang, J-s., and J. D. Sipe.** Recombinant human serum amyloid A ($apoSAA_p$) binds cholesterol and modulates cholesterol flux. *J. Lipid Res.* 1995. **36:** 37–46.

Supplementary key words amyloidosis • amyloid A (AA) • acute phase response

The acute phase response proteins serum amyloid A (apoSAA) are injury specific apolipoproteins, synthesized mainly in liver under cytokine regulation and released into the bloodstream as constituents of high density lipoproteins (HDL) during disturbances of homeostasis (1). Four human SAA gene loci have been described on chromosome 11; the acute phase apoSAA isoforms are encoded by two genes SAA_1 and SAA_2 with allelic variants described for both sites; the locus designated SAA_3 appears to be a pseudogene, and the SAA_4 locus encodes a constitutively expressed $apoSAA_4$ isoform that is present on HDL during homeostasis and does not vary significantly during the acute phase response (2–10). The $apoSAA_1$ and $apoSAA_2$ content of HDL may increase by 1000-fold after tissue injury and cell necrosis and, at points, these acute phase apoSAA isoforms can constitute as much as 80% of total HDL proteins, presumably by displacement of apoA-I (11–13). The half-life of the total population of acute phase apoSAA isoforms has been estimated to be less than 24 h, whereas apoA-I has a half-life of approximately 4–6 days. This difference in clearance times between apoSAA and apoA-I implies that either apoSAA proteins are dissociated from HDL before clearance from plasma or they are associated with a subspecies of HDL particle with a metabolic fate different from HDL lacking apoSAA (14). In vitro, peritoneal macrophages bind 6- to 8-fold more apoSAA-rich HDL than normal HDL (15) and resident peritoneal cells including macrophages selectively degrade apoSAA from HDL (16). Steinmetz and coworkers (17) reported that apoSAA modulates lecithin:cholesterol acyltransferase (LCAT) activity associated with HDL.

Recent studies of experimental inflammation in Syrian hamsters suggest that apoSAA may alter lipid metabolism during the acute phase response. The concentration of apoSAA on HDL is maximal at 36 h after inflammatory stimulation, preceding increases in plasma cholesterol and triglyceride concentrations, which are maximal at 48 and 60 h, respectively (18, 19). At the time of maximal plasma cholesterol concentration, HDL cholesterol is decreased, LDL cholesterol is increased, and hepatic secretion of cholesterol is decreased, all of which suggest a decreased clearance of cholesterol.

Abbreviations: AA, amyloid A; apoSAA, serum amyloid A; apoA-I, apolipoprotein A-I; HDL, high density lipoprotein; NHDL, normal high density lipoprotein; PC, 1.-α-phosphatidylcholine.

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Until now, although the potential role of apoSAA in modulation of cholesterol transport or metabolism has been recognized, evidence for a direct interaction of apoSAA with cholesterol has been lacking. In this study, using recombinant generated apoSAA, we have demonstrated equimolar binding of cholesterol by $apoSAA_p$ and an increase in size upon cholesterol binding. Cholesterol flux in solution is enhanced by $apoSAA_p$. Uptake of cholesterol by HepG2 cells is enhanced by $apoSAA_p$, which appears to be catabolized by cell-associated enzymes. These findings suggest that during inflammation apoSAA may modulate cholesterol transport by serving as a cholesterol binding protein.

MATERIALS AND METHODS

Materials

Recombinant synthetic human $apoSAA_p$, was purchased from Pepro Tech (Rocky Hill, NJ). $ApoSAA_p$ is a hybrid molecule corresponding to human $apoSAA_{1\alpha}$ except for the N-terminal methionine and substitution of asparagine for aspartic acid at position 60 and arginine for histidine at position 71; the latter two substituted residues are present in $apoSAA_{2\beta}$ (**Fig. 1**). The lyophilized $apoSAA_p$ was dissolved in water at concentrations of 100 μg/ml (used within 2 months) and 1000 μg/ml (used within 2 weeks), and stored at −20°C. Immediately before use, the stock solution was diluted with serum-free RPMI medium.

$Na^{125}I$, [4-^{14}C]cholesterol (57.1 mCi/mmol), [1,2,6,7-^{3}H(N)]cholesterol (101 Ci/mmol), [dipalmitoyl-1-^{14}C]-phosphatidylcholine (115 mCi/mmol), and Gel and Tissue Solubilizer were purchased from DuPont New England Nuclear (Boston, MA). L-α-phosphatidylcholine (PC) (type III: from bovine liver), cholesterol, bovine serum albumin (BSA), tissue culture medium, and fetal calf serum were purchased from Sigma Chemical Co., St. Louis, MO. PC and cholesterol were dissolved in chloroform–methanol 3:1 and ethanol at concentration of 10 mg/ml, respectively, and stored at −20°C. All other chemicals were reagent grade.

Rabbit anti-human cathepsin G serum was purchased from Calbiochem Corporation, La Jolla, CA. Rabbit anti-human SAA polyvalent serum and human apoA-I were kindly provided by Dr. F. C. de Beer, University of Kentucky College of Medicine, Lexington, KY.

Gradient gels, 4–20%, were purchased from Bio-Rad Laboratories (Melville, NY). Sephadex G-25, Sephacryl S-100, and a low molecular weight gel filtration calibration kit were obtained from Pharmacia (Piscataway, NJ).

Normal high density lipoprotein (NHDL) was isolated by ultracentrifugation from plasma obtained from normal Syrian hamsters (13); the preparation consisted of 47% protein, 27% phospholipid, and 26% cholesterol.

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                    10                  20                  30
apoSAA1α  RSFFSFLGEA FDGARDMWRA YSDMREANYI
apoSAA2β  ---------- ---------- ----------
apoSAAp   ---------- ---------- ----------
                    40                  50                  60
apoSAA1α  GSDKYFHARG NYDAAKRGPG GVWAAEAISD
apoSAA2β  ------ -- -- --- -- --- - - - A - - - V - - N
apoSAAp   ------ -- -- --- -- --- - - - V - - - A - - N
                    70                  80                  90
apoSAA1α  ARENIQRFFG HGAEDSLADQ AANEWGRSGK
apoSAA2β  - ------ LT - R- --- ----- --- K -- --- R
apoSAAp   ---- --- FF - R- --- ----- --- E- - --- K
                   100
apoSAA1α  DPNHFRPAGL PEKY
appSAA2β  ---- ---- -- ----
apoSAAp   ------ ---- ----
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Fig. 1. Human apoSAA isoforms. Recombinant synthetic human $apoSAA_p$ is a hybrid molecule corresponding to human $apoSAA_{1\alpha}$ (5, 6) except for the N-terminal methionine and substitution of asparagine for aspartic acid at position 60 and arginine for histidine at position 71; the latter two substituted residues are present in $apoSAA_{2\beta}$ (31).

Biochemical assays

Protein was determined colorimetrically using a kit purchased from Pierce Chemical (Rockford, IL). Cholesterol was measured using a kit purchased from Sigma Chemical Co., St. Louis, MO and phospholipid by the method of Rouser, Siakotos, and Fleischer (20).

Cholesterol binding assay

To measure the amount of cholesterol bound by $apoSAA_p$, [^{3}H]cholesterol in quantities from 25 pmol to 1.25 nmol were added to glass tubes and the solvent was evaporated under a stream of N_2. $ApoSAA_p$, 2 μg or 167 pmol, was added in 1 ml of serum-free RPMI medium and mixed with cholesterol using a vortex mixer. The mixtures were incubated at 25°C for 24 h. Preliminary experiments established that about 90% of the lipid tracer was desorbed from the glass tube surface after 24 h incubation at 25°C in the presence of $apoSAA_p$. Cholesterol-$apoSAA_p$ complexes were isolated by rapid filtration through nitrocellulose membranes (0.45 μm, Millipore Corp, Bedford, MA) previously soaked for at least 4 h in phosphate-buffered saline (PBS) containing 0.001% cholesterol. More than 92% of $apoSAA_p$ is bound to nitrocellulose membrane. The filters were washed ten times with 10 ml of ice-cold PBS, dried at room temperature, and the quantity of bound cholesterol was determined by liquid scintillation counting with an efficiency of 89%. The data are expressed as the molar ratio of cholesterol bound to $apoSAA_p$.

Labeling of $apoSAA_p$ and apoA-I with ^{125}I

$ApoSAA_p$ and apoA-I, each at a concentration of 1 mg/ml in 0.15 M NaCl, pH 7.4, were combined with ICl reagent in 0.1 M glycine. A stock solution of ICl was prepared by dissolving 100 mg of $NaIO_4$ in 2 ml H_2O, 150 mg of NaI in 6 ml of 6 N HCl, and adding the $NaIO_4$

solution to the NaI solution, followed by the addition of 32 ml of H_2O and 5 ml of CCl_4. N_2 was bubbled through the solution prior to storage at 4°C. Immediately before use, the stock solution was diluted 1:10 with H_2O. After reaction for 10 min with 1 mCi Na ^{125}I at room temperature, the free iodide was removed by gel filtration using Sephadex G-25. The specific radioactivities of ^{125}I-labeled $apoSAA_p$ and ^{125}I-labeled apoA-I were 0.35 mCi/mg and 0.8 mCi/mg, respectively.

Gel filtration

$ApoSAA_p$ and other proteins, and cholesterol and PC, alone or in the presence of protein, were fractionated on a column (1.5 × 100 cm) of Sephacryl-S-100 previously equilibrated with PBS (pH 7.2). Fractions of 2.8 ml were collected at a flow rate of 0.8 ml/min and were analyzed by measurement of absorbance at 280 nm and/or by gamma or liquid scintillation counting. Albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa) purchased as a low molecular weight calibration kit were used for molecular weight determination. Under a stream of N_2, 10 nmol of cholesterol and/or PC with or without [^{3}H]cholesterol and/or [^{14}C]PC were dried in a borosilicate glass tube followed by addition of 1 ml serum-free RPMI medium containing 2 μg (167 pmol) $apoSAA_p$ and mixing by vortexing, followed by incubation at 25°C for 24 h. ^{125}I-labeled $apoSAA_p$ was mixed together with either unlabeled cholesterol or phospholipid to determine size changes after lipid binding.

Adsorption of ^{125}I-labeled $apoSAA_p$ to polystyrene microtiter wells

^{125}I-labeled $apoSAA_p$ was added at various concentrations in serum-free RPMI medium. Triplicate aliquots were incubated in microtiter wells at 37°C for up to 6 h. The amount of ^{125}I-labeled $apoSAA_p$ remaining in solution at various intervals was determined by counting triplicate 10-μl aliquots. After incubation was completed, all of the remaining medium was removed, the wells were washed three times, and the amount of adsorbed ^{125}I-labeled $apoSAA_p$ was determined by counting individual wells.

Inhibition and reversal of ^{125}I-labeled $apoSAA_p$ adsorption by lipids and lipoproteins

Cholesterol and PC were diluted in serum-free RPMI medium (pH 7.2) at varying concentrations immediately prior to use; however, the concentration of their respective solvents in the medium was constant (0.4%) and the same volume of solvent was used as a control. At this concentration, solubilization of $apoSAA_p$ by solvent was not observed.

To measure inhibition of $apoSAA_p$ adsorption, 100-μl aliquots of ^{125}I-labeled $apoSAA_p$ (2 μg/ml) in RPMI medium with varying concentrations of cholesterol, PC, or NHDL were added to 96-well polystyrene plates, incubated at 37°C for 2 h, then 10 μl of triplicate aliquots were removed for counting of radioactivity. To measure the reversal of the inhibitory effect of cholesterol by 3 M KBr, PL and NHDL on ^{125}I-labeled $apoSAA_p$ adsorption, 3 M KBr was added to RPMI (pH 7.2) or to 0.1 M sodium bicarbonate buffer (pH 9.6).

To measure reversal of adsorption, i.e., the release of bound ^{125}I-labeled $apoSAA_p$ from microtiter wells by lipid and lipoprotein or antibodies, 100 μl of cholesterol, PC, or NHDL at varying concentrations in serum-free RPMI medium was added to wells to which ^{125}I-labeled $apoSAA_p$ had been adsorbed and, after the removal of the coating medium, washed three times with RPMI lacking apoSAA. After incubation at 37°C for 2 h, triplicate 10-μl aliquots were removed for counting of released ^{125}I-labeled $apoSAA_p$.

[^{14}C]cholesterol exchange

Under a stream of N_2, 2.5 nmol of [^{3}H]cholesterol, [^{14}C]cholesterol, or [^{14}C]PC, or [^{3}H]cholesterol and [^{14}C]PC together were dried in borosilicate glass tubes. Then 5 ml of NHDL (4.7 μg protein, 2.7 μg phospholipid, and 2.6 μg cholesterol) in serum-free RPMI medium was added and mixed by vortexing. Four-tenths ml of the resultant radiolabeled NHDL solution was added to dialysis bags that retained molecules with MW greater than 3500. The bags were placed in quadruplicate wells of a 24-well tissue culture plate containing $apoSAA_p$, apoA-I, or albumin in RPMI medium and incubated at 37°C. After 1 h, duplicate 5-μl aliquots were removed from each well for counting of radioactivity. Preliminary experiments established that exchange was >90% complete by 1 h. During this time, about 6% of [^{3}H]cholesterol and 5% of [^{14}C]PC were removed from solution by adherence to the dialysis membrane.

[^{14}C]cholesterol uptake (influx) by HepG2 cells

HepG2 cells were propogated in monolayer culture at 37°C, 5% CO_2 in 24-well microtiter plates using RPMI 1640 medium containing 2 mM glutamine plus 10% FCS and 50 μg/ml gentamycin. After washing thrice with serum-free RPMI medium, 0.2 ml medium containing [^{14}C]cholesterol alone or with different concentrations of $apoSAA_p$, BSA, or antibodies to apoSAA or to cathepsin G were added to each of triplicate wells and incubated for 24 h. After incubation, the culture medium was removed, each well was washed three times with serum-free RPMI medium, 0.5 ml of Gel and Tissue Solubilizer was added, followed by incubation at 70°C for 4 h. Triplicate

50-μl aliquots were removed for counting of radioactivity and triplicate 10-μl aliquots for protein assay using the BSA protein assay reagent according to the manufacturer's instructions. Cell viability remained constant throughout the incubation period as assessed by Trypan blue exclusion.

Association of ^{125}I-labeled apoSAA$_p$ with HepG2 cells

After washing HepG2 monolayers, propogated as described above, with serum-free RPMI medium thrice, 0.4 ml of medium containing ^{125}I-labeled apoSAA$_p$ or ^{125}I-labeled apoA-I was added to each of quadruplicate wells for each time point and incubated at 37°C in 5% CO_2 for up to 20 h. The amount of ^{125}I-labeled apoSAA$_p$ remaining in the culture medium at various intervals was determined by counting duplicate 100-μl pooled aliquots of medium. One-ml aliquots of medium were also fractionated by gel filtration. After the last incubation period, all of the remaining medium was removed, the wells were washed thrice, and 0.5 ml of Gel and Tissue Solubilizer was added to each well and incubated at 70°C for 4 h. Duplicate, pooled 100-μl aliquots were removed for counting of radioactivity.

^{125}I-labeled apoSAA$_p$ catabolism

Peritoneal exudate cells from Syrian hamsters and HepG2 cells were plated into 96-well tissue culture plates at densities of 5 × 10^4 to 1 × 10^5 cells/well in 0.2 ml of RPMI medium. One hundred twenty ng ^{125}I-labeled apoSAA$_p$ was added to each well and incubated at 37°C, 5% CO_2 for 24 h. Culture supernatants were collected and 15-μl aliquots were analyzed by electrophoresis on 4–20% gradient SDS-PAGE gels. Conditioned media from 24 h PEC cultures and 72 h HepG2 cell cultures were also incubated with ^{125}I-labeled apoSAA$_p$ to determine whether the proteolytic activity was cell-associated or was secreted into the culture medium.

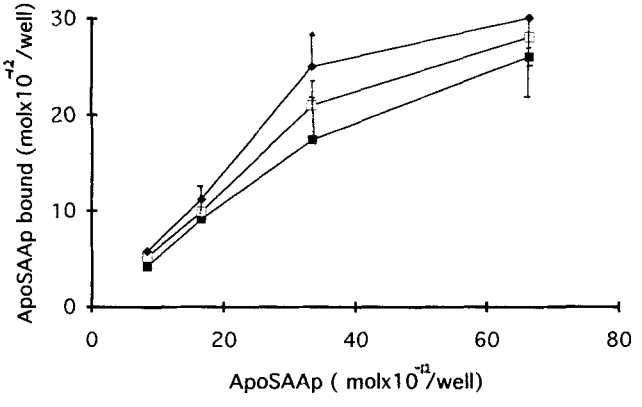

Fig. 2. Time course of ^{125}I-labeled apoSAA$_p$ adsorption from RPMI to polystyrene microtiter wells. One hundred μl of ^{125}I-labeled apoSAA$_p$ in serum-free RPMI medium at the concentrations indicated was added to each well and incubated at 37°C. At intervals of 2 (■), 4 (□), and 6 (♦) h, the medium was removed and each well was washed with H_2O three times. Each well was cut off for counting of radioactivity. Each value is the mean ± standard deviation (SD), n = 6.

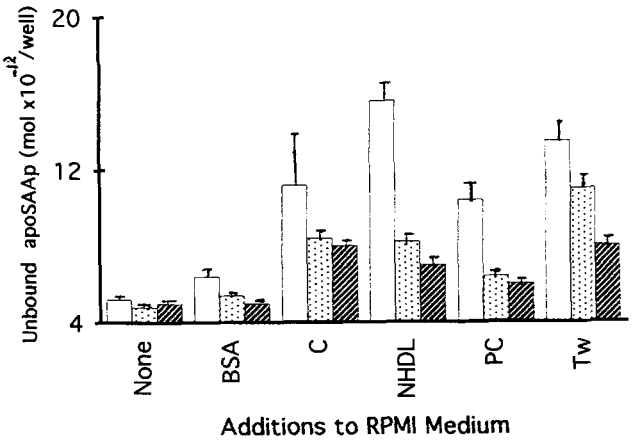

Fig. 3. Reversal of cholesterol (C), PC, and NHDL solubilization of ^{125}I-labeled apoSAA$_p$ by 3 M KBr. ^{125}I-labeled apoSAA$_p$ was diluted in RPMI medium, pH 7.2 (□), 3 M KBr in RPMI medium, pH 7.2 (▨), and 3 M KBr in buffer, pH 9.6 (▨), respectively, (2 μg/ml). As indicated, ^{125}I-labeled apoSAA$_p$ with BSA (40 μg/ml), C (40 μg/ml), PC (40 μg/ml), NHDL (40 μg/ml), or Tween-20 (Tw) (2%) were added to each well; the final volume was 100 μl per well. After 2 h incubation at 37°C, triplicate aliquots (10 μl) were removed for counting of radioactivity. Each value is the mean ± SD, n = 6.

RESULTS

Adsorption of ^{125}I-labeled apoSAA$_p$ by polystyrene microtiter wells

Within 6 h of incubation at 37°C in polystyrene microtiter wells, as much as 80–90% of ^{125}I-labeled apoSAA$_p$ was removed from serum-free RPMI medium, while the concentration of human ^{125}I-labeled apoA-I in RPMI medium was essentially unchanged. The cleared apoSAA$_p$ was recovered tightly bound to microtiter wells (**Fig. 2**). This adsorption of apoSAA$_p$ by microtiter wells was diminished in the presence of cholesterol, PC, or HDL at concentrations ranging from 5 to 80 μg/ml. However, apoSAA$_p$ adsorption was not affected by the presence of BSA over the concentration range 10 to 50 μg/ml, nor was it affected by the presence of lipid solvent only, nor by pretreatment of the microtiter wells with cholesterol, 40 μg/ml. These results suggest that it is the interaction of apoSAA$_p$ with cholesterol and phospholipids in solution that enhances its solubility and/or diminishes its adsorption. Adsorption of apoSAA$_p$ in the presence of lipids was significantly enhanced by the presence of 3 M KBr (**Fig. 3**). Adsorbed apoSAA$_p$ was partially released upon introduction of RPMI containing cholesterol, PC, or NHDL at concentrations of 5–80 μg/ml to wells after removal of ^{125}I-labeled apoSAA$_p$

coating medium and washing of wells (**Fig. 4A**). Adsorbed apoSAA$_p$ was released upon incubation with anti-apoSAA antiserum, but not by control anti-cathepsin G antiserum (Fig. 4B). However, incubation with apoSAA$_p$ did not result in release of ^{125}I-labeled apoSAA$_p$, suggesting that the adsorbed protein is tightly bound and not freely exchangeable.

Enhancement of cholesterol flux from HDL by apoSAA$_p$

The presence of apoSAA$_p$ in RPMI enhanced the diffusion of cholesterol from HDL across a dialysis membrane that retains molecules of MW greater than 3500 by as much as 5-fold, whereas exchange in the presence of phospholipid was approximately 3-fold relative to RPMI alone. ApoSAA$_p$ enhanced cholesterol flux to a significantly greater extent than did apoA-I or BSA (**Table 1**, and **Table 2**). Approximately 1 mole of cholesterol and 0.24 mole of phospholipid diffused across the membrane per mole of apoSAA$_p$ in solution on the other side of the membrane.

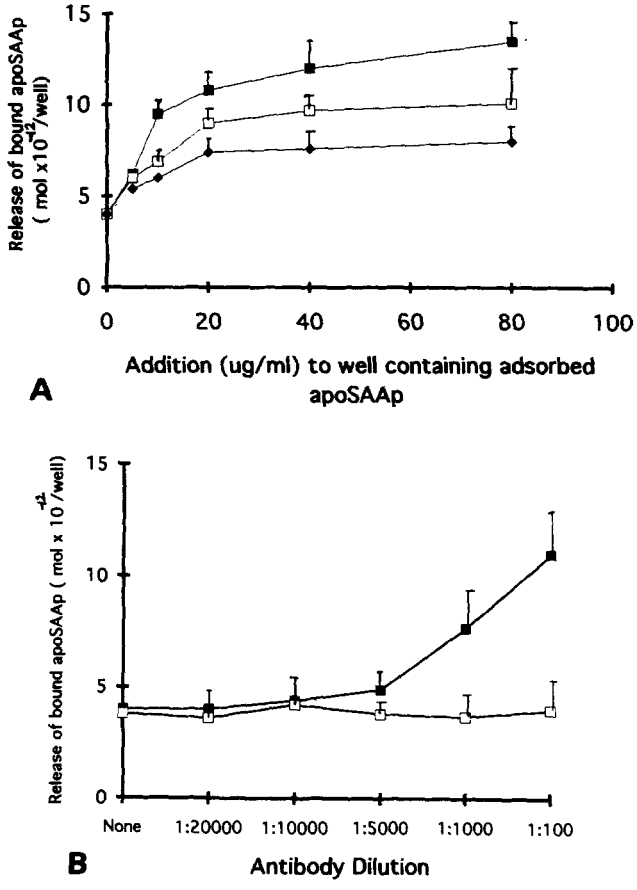

Fig. 4. A: Release of bound ^{125}I-labeled apoSAA$_p$ by lipids and lipoproteins. Bound ^{125}I-labeled apoSAA$_p$ was prepared as described in Materials and Methods. One hundred μl of cholesterol (C, □), PC (♦), or NHDL (■) at the concentrations indicated in serum-free RPMI medium was added to ^{125}I-labeled apoSAA$_p$ bound microtiter wells and incubated at 37°C for 2 h. Triplicate aliquots (10 μl) were removed for counting of radioactivity. B: Release of bound ^{125}I-labeled apoSAA$_p$ by antibodies to apoSAA. Bound ^{125}I-labeled apoSAA$_p$ was prepared as described in Materials and Methods. Antibodies to apoSAA (■) or antibodies to cathepsin G (□) in serum-free RPMI medium were added to ^{125}I-labeled apoSAA$_p$ bound microtiter wells, incubated at 37°C for 2 h. Triplicate aliquots (10 μl) were removed for counting of radioactivity. Each value is the mean ± SD, n = 6.

TABLE 1. ApoSAA$_p$ enhances [^{14}C]cholesterol diffusion from HDL into RPMI

Addition	Cholesterol Flux
	pmol/ml
None	39 ± 4
ApoSAA$_p$ (2 μg/ml)	179 ± 19
ApoSAA$_p$ (4 μg/ml)	347 ± 23
ApoA-I (2 μg/ml)	131 ± 9
ApoA-I (6 μg/ml)	136 ± 7
BSA (2 μg/ml)	43 ± 7
FBS (2 μg/ml)	49 ± 5

Under nitrogen, [^{14}C]cholesterol (2.5 nmol) was dried in a borosilicate glass tube, then 5 ml of NHDL (4.7 μg protein, 2.7 μg phospholipid, and 2.6 μg cholesterol) in serum-free RPMI was added to the tube, and mixed by vortexing. Aliquots (0.4 ml) of the [^{14}C]cholesterol–NHDL medium were added to dialysis bags that retained molecules with MW greater than 3500; the bags were placed in duplicate wells of a 24-well tissue culture plate containing the indicated proteins in RPMI medium and incubated at 37°C. At intervals of 1 h, duplicate 5 μl-aliquots of medium were removed for counting of radioactivity. Each value is the mean ± SD, n = 4.

Cholesterol binding by apoSAA$_p$

An assay was developed to measure [^{3}H]cholesterol binding by apoSAA. The [^{3}H]cholesterol complexes were isolated and quantified as described in Materials and Methods. More than 75% of [^{3}H]cholesterol binding to apoSAA$_p$ can be inhibited by unlabeled cholesterol at concentrations ranging from 25 nM to 125 μM (**Fig. 5**), but less than 15% of [^{3}H]cholesterol binding to apoSAA$_p$ was inhibited by unlabeled PC (1–125 μM) (J-s. Liang and J. D. Sipe, unpublished observations). The K_d for cholesterol binding is 1.7 ± 0.3 × 10^{-7} M and B_{max} is 1.1 ± 0.1 mol/mol (Fig. 5).

Increase in apoSAA$_p$ size after cholesterol binding

Fractionation of ^{125}I-labeled apoSAA$_p$, at 2 μg/ml, by gel filtration resulted in a single peak maximally eluted in fraction 60. However, after incubation of ^{125}I-labeled apoSAA$_p$ for 24 h at 25°C with cholesterol (10 nmol/ml) that had been previously dried under nitrogen, ^{125}I-labeled apoSAA$_p$ was maximally eluted 7 fractions earlier in fraction 53 (**Fig. 6A**). After a similar incubation with PC (10 nmol/ml) that had been previously dried under nitrogen, ^{125}I-labeled apoSAA$_p$ was eluted 4 fractions earlier in fraction 56. (Fig. 6B), and when incubated with both cholesterol and PC, ^{125}I-labeled apoSAA$_p$ was

TABLE 2. ApoSAA$_p$ enhances a higher ratio of cholesterol to phospholipid (PL) diffusion from HDL

Addition	PL or C Diffusion from NHDL to RPMI			
	[^{14}C]PL-NHDL PL	[^{3}H]C-NHDL C	[^{14}C]PL + [^{3}H]C-NHDL PL	[^{14}C]PL + [^{3}H]C-NHDL C
	pmol/ml			
None	15 ± 2	33 ± 3	17 ± 1	38 ± 2
ApoSAA$_p$ (167 pmol, 2 μg/ml)	40 ± 1	163 ± 3	31 ± 3	170 ± 5
BSA (30 pmol, 2 μg/ml)	16 ± 2	47 ± 5	14 ± 1	43 ± 4

Under nitrogen, [^{14}C]PC and [^{3}H]C (2.5 nmol) were dried in a borosilicate glass tube as indicated, then 5 ml of NHDL (4.7 μg protein, 2.7 μg phospholipid, and 2.6 μg cholesterol) in serum-free RPMI medium was added to the tube, and mixed by vortexing. Aliquots (0.4 ml) of the above complexes were added to dialysis bags that retained molecules with MW greater than 3500; the bags were placed in quadruplicate wells of a 24-well tissue culture plate containing the indicated proteins in RPMI medium and incubated at 37°C. At intervals of 1 h, duplicate 5-μl aliquots of medium were removed for counting of radioactivity. Each value is the mean ± SD, n = 4.

eluted 9 fractions earlier, in fraction 51 (Fig. 6C). Incubation of [^{3}H]cholesterol with apoSAA$_p$ (2 μg/ml, 167 pmol), resulted in its coelution with apoSAA$_p$, approximately 25 fractions ahead of [^{3}H]cholesterol in the absence of apoSAA$_p$ (Fig. 6D). These data suggest that the direct interaction of apoSAA$_p$ with cholesterol either increased the size by dimerization or changed the conformation of apoSAA$_p$ such that it was eluted at the position where a dimer would be eluted. The apparent molecular weight of the complex was calculated to be 23 kDa as determined by the elution volume relative to elution volumes of molecular weight standard proteins.

Enhancement of [^{14}C]cholesterol uptake by HepG2 cells in the presence of apoSAA$_p$

Confluent HepG2 cells monolayers were washed thrice with Hank's balanced salt solution (HBSS) and incubated with [^{14}C]cholesterol in serum-free medium. [^{14}C]cholesterol uptake was enhanced by and was dose dependent on apoSAA$_p$ over the range 2–40 μg/ml (0.17–3.3 μM). BSA at a concentration of 10 μg/ml had no effect (**Fig. 7**). The enhancement by apoSAA$_p$ of [^{14}C]cholesterol uptake by HepG2 cells was completely blocked by antibodies to apoSAA (1:5000 to 1:20000), not by corresponding quantities of rabbit anti-human cathepsin G (**Table 3**). A size change of ^{125}I-labeled apoSAA$_p$ was not observed after incubation of ^{125}I-labeled apoSAA$_p$ with HepG2 cells for 1, 2, and 6 h and analysis of culture supernatants by gel filtration. This suggested that apoSAA$_p$ does not extract much cell-associated lipid to form SAA-lipid complexes from HepG2 cells during this incubation time.

ApoSAA$_p$ uptake by HepG2 cells

The percentage of the total ^{125}I-labeled apoSAA$_p$ radioactivity detectable in HepG2 cells increased for up to 6 h after its addition; correspondingly there was a decrease in the percentage of total ^{125}I-labeled

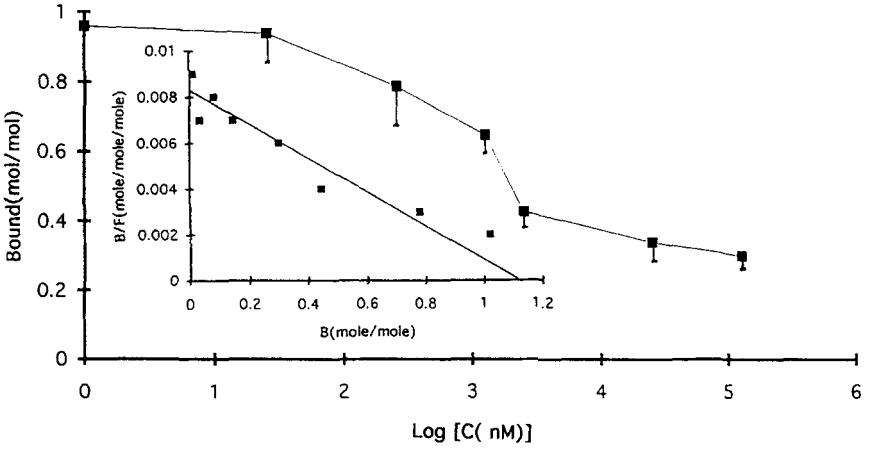

Fig. 5. Inhibition of binding of [^{3}H]cholesterol to apoSAA$_p$ by different concentrations of unlabeled cholesterol. ApoSAA$_p$ (167 pmol) was incubated with 250 pmol [^{3}H]cholesterol and varying concentrations of unlabeled cholesterol as indicated. The inset shows a Scatchard plot. Data are expressed as the number of moles of [^{3}H]cholesterol bound per mole of apoSAA$_p$. Each value is the mean ± SD, n = 6.

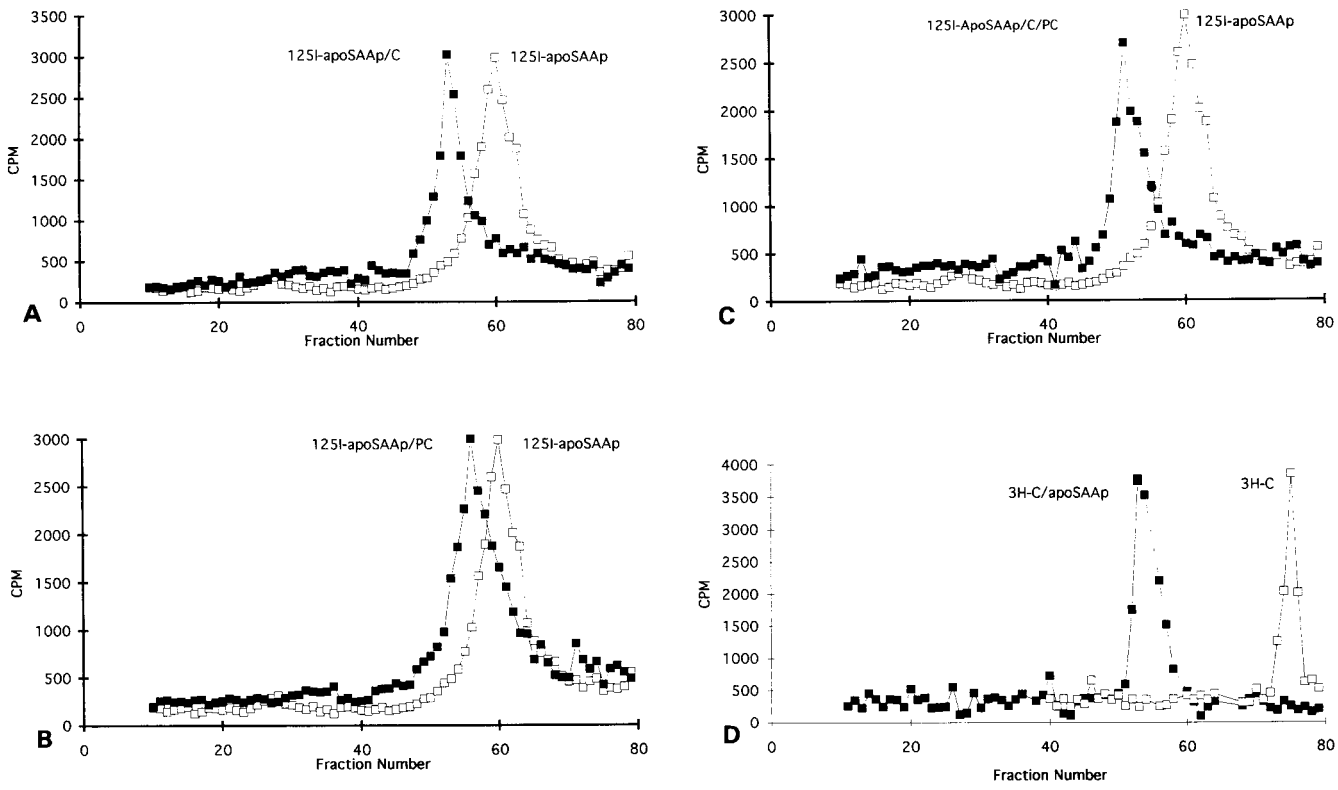

Fig. 6. Gel chromatography of ^{125}I-labeled apoSAA$_p$ or apoSAA$_p$ after incubation at 25°C for 24 h with cholesterol (C) or [^{3}H]cholesterol and PC. Under a stream of N_2, 10 nmol of cholesterol and/or PC with or without [^{3}H]cholesterol was dried in a borosilicate glass tube, followed by addition of 1 ml serum-free RPMI containing 2 μg (167 pmol) apoSAA$_p$ and mixing by vortexing, followed by incubation at 25°C for 24 h. After incubation, the mixtures of ^{125}I-labeled apoSAA$_p$ and cholesterol (A) or PC (B) or cholesterol and PC (C) or unlabeled apoSAA$_p$ incubated with [^{3}H]cholesterol (D) were fractionated on a column (1.5 × 100 cm) of Sephacryl S-100 previously equilibrated with PBS (pH 7.2) to determine size changes after lipid binding.

apoSAA$_p$ radioactivity present in the culture medium (**Fig. 8**). No intact apoSAA$_p$ was detectable in the lysed cellular fraction. By 20 h, the amount of cell-associated ^{125}I-labeled apoSAA$_p$ was diminished, while the total radioactivity in the culture medium increased. The total radioactivity, i.e., the sum of cell-associated and supernatant radioactivity, did not change. The amount of intact ^{125}I-labeled apoSAA$_p$ was in fact decreased (**Fig. 9**), suggesting intracellular or membrane-associated degradation of ^{125}I-labeled apoSAA$_p$. Incubation of apoSAA$_p$ with conditioned medium from HepG2 cells, in contrast to cell cultures, did not result in catabolism (J-s. Liang and J. D. Sipe, unpublished observations).

Catabolism of apoSAA$_p$

In order to determine how apoSAA$_p$ might be interacting with HepG2 cells to enhance cholesterol uptake, the fate of ^{125}I-labeled apoSAA$_p$ after incubation with

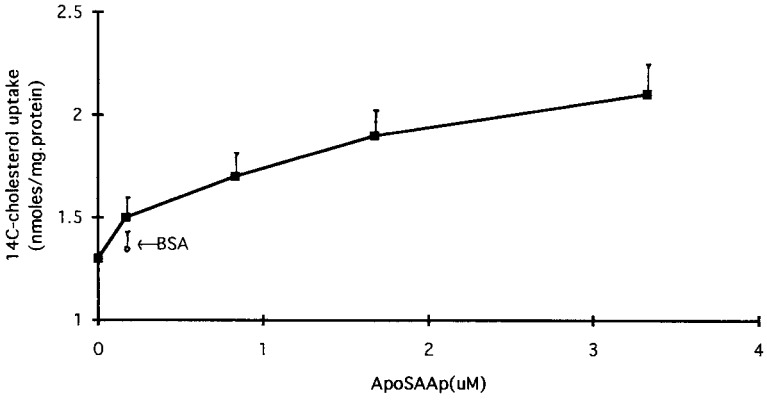

Fig. 7. Effect of apoSAA$_p$ on [^{14}C]cholesterol uptake by HepG2 cells. After washing the cells thrice with serum-free RPMI medium, 200 μl medium containing 0.85 nmol of [^{14}C]cholesterol alone or with different concentrations of apoSAA$_p$ as indicated or BSA (10 μg/ml) was added to each well and incubated for 24 h. After incubation, the culture medium was removed; each well was washed three times with serum-free medium, and then 0.5 ml of Gel and Tissue Solubilizer was added to each well, followed by incubation at 70°C for 4 h. Triplicate aliquots (50 μl) were removed for counting of radioactivity, and triplicate aliquots (10 μl) were removed for protein assay. Each value is the mean ± SD, n = 4.

TABLE 3. Antibodies block apoSAA$_p$ enhancement of [^{14}C]cholesterol uptake by HepG2 cells

Addition	[^{14}C]C Uptake by HepG2 cells
	nmol/mg protein
None	0.9 ± 0.03
ApoSAA$_p$ (10 μg/ml)	1.6 ± 0.09
BSA (10 μg/ml)	0.9 ± 0.03
Anti-SAA AB (1:10,000)	0.8 ± 0.02
Anti-Ca AB (1:5,000)	0.9 ± 0.04
ApoSAA$_p$ + anti-Ca (1:5,000)	1.7 ± 0.08
ApoSAA$_p$ + anti-SAA	
1:5,000	1.0 ± 0.01
1:10,000	1.4 ± 0.02
1:20,000	1.7 ± 0.02

After washing thrice with serum-free RPMI medium, 200 μl medium containing [^{14}C]cholesterol alone or with different concentrations of apoSAA$_p$, BSA, or antibodies to apoSAA or control antibodies were added to each well and incubated for 24 h. After incubation, the culture medium was removed, each well was washed three times again, and then 0.5 ml of Gel and Tissue Solubilizer was added to each well, followed by incubation at 70°C for 4 h. Triplicate 50-μl aliquots were removed for counting of radioactivity and triplicate 10-μl aliquots for protein assay. Rabbit anti-human cathepsin G (Ca) was used as control antibody. Each value is the mean ± SD, n = 4.

HepG2 cells was compared with that after incubation with peritoneal exudate cells (PEC) from hamsters undergoing an acute phase response. After 24 h incubation with HepG2 cells, apoSAA$_p$ was diminished in quantity, but appeared to be intact, while no apoSAA$_p$ remained after incubation with PEC (Fig. 9). Results using conditioned medium from PEC cultures were similar to those observed with cells in culture while apoSAA$_p$ was not diminished upon incubation with conditioned medium from HepG2 cells (J-s. Liang and J. D. Sipe, unpublished observations).

DISCUSSION

The inverse correlation between the amount of plasma HDL cholesterol and coronary artery disease has been widely studied (21). During the course of an inflammatory episode, apoSAA$_1$ and apoSAA$_2$ can transiently constitute as much as 80% of total HDL proteins, reaching concentrations greater than 1 mg/ml and displacing the primary protein constituent of HDL, apoA-I (11, 13). ApoSAA$_1$ and apoSAA$_2$ isoforms have been shown to be cleared more rapidly than apoA-I and may be dissociated from HDL before catabolism (14).

HDL are believed to be the physiological acceptors of cholesterol. All of the apolipoproteins A are characterized by the presence of amphipathic helixes that have been shown to be responsible for the association of these apolipoproteins with lipids (22). Variations in the number and structure of such amphipathical helical regions within a specific apolipoprotein result in differing affinities for lipids (23). In one model of cholesterol flux, the cholesterol acceptor lipoprotein particles are thought to be loosely and transiently anchored to the areas of plasma membranes through the association of amphipathic helixes with cell membrane lipids, thereby completing the process of cholesterol flux between HDL and cells (23, 24).

Like other HDL apolipoproteins, apoSAA contains amphipathic helical regions. The amphipathic properties of the first 26 amino acids of a 45 amino acid amino-terminal amyloid A (AA) fragment of human apoSAA

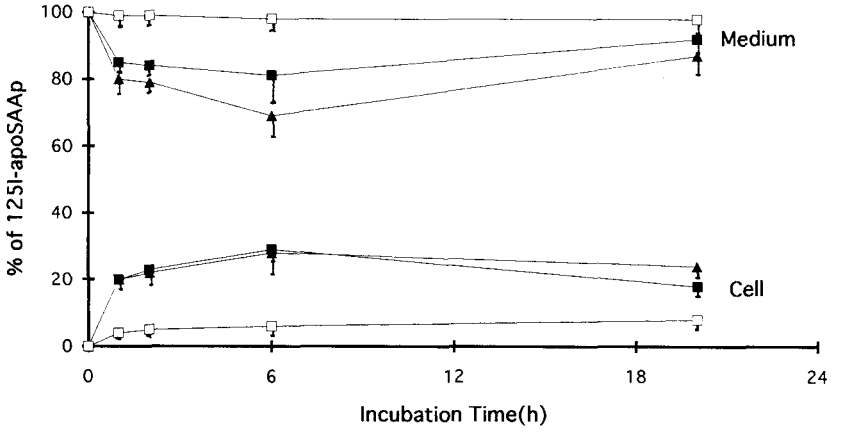

Fig. 8. ^{125}I-labeled apoSAA$_p$ uptake by HepG2 cells. After washing with serum-free RPMI medium three times, 400 μl of the medium containing ^{125}I-labeled apoSAA$_p$, 0.04 μM (■); 0.13 μM (▲); or ^{125}I-labeled apoA-I, 0.06 μM (□) was added to each of quadruplicate wells for each of four time points (1, 2, 6, and 20 h) and incubated at 37°C in 5% CO_2 for up to 20 h. The amount of ^{125}I-labeled apoSAA$_p$ remaining in the medium at various intervals was determined by counting duplicate pooled aliquots (100 μl) of medium. At each time point, all of the remaining medium was removed, the wells were washed three times, and 0.5 ml of Gel and Tissue Solubilizer was added to each well and incubated at 70°C for 4 h. Duplicate pooled aliquots (100 μl) were removed for counting of radioactivity. Each value is the mean ± SD, n = 4.

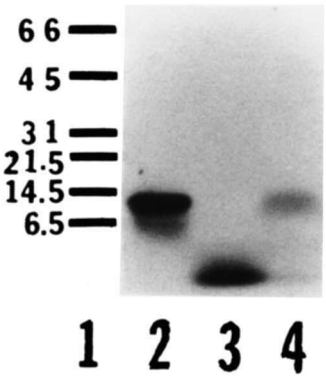

Fig. 9. Cellular differences in apoSAA$_p$ degradation. Peritoneal exudate cells from Syrian hamsters and HepG2 cells were plated into 96-well tissue culture plates at densities of 5 × 10^4 to 1 × 10^5 cells/well in 0.2 ml of RPMI. ^{125}I-labeled apoSAA$_p$ (120 ng) was added to each well and incubated at 37°C in 5% CO_2 for 24 h. Culture supernatants were pooled and 15-μl aliquots were used for analysis by electrophoresis on 4–20% gradient SDS-PAGE gels. 1: Markers; 2: apoSAA$_p$, starting material; 3: apoSAA$_p$ incubated with PEC for 24 h; 4: apoSAA$_p$ incubated with HepG2 cells for 24 h.

were described (25). The first 11 residues of the amino-terminal peptide are all strongly hydrophobic and very important to lipid binding (26, 27). An increase in alpha helical content in one human apoSAA isoform upon phospholipid binding was observed, but the ability of lipid free apoSAA to associate directly with cholesterol has not been investigated (28). In this study, instead of using natural apoSAA monomers, recombinant human apoSAA$_p$, a hybrid of naturally occurring human apoSAA$_1$ and apoSAA$_2$, was used (Fig. 1). ApoSAA preparations isolated from plasma are heterogenous due to the presence of multiple gene products and their posttranslational modifications; it is difficult to separate apoSAA isoforms and they may be expected to contain variable amounts of bound lipids that would be difficult to characterize.

A cholesterol-apoSAA binding assay was developed to demonstrate the direct interaction of apoSAA with cholesterol, and apoSAA-cholesterol complexes were isolated by gel filtration (Figs. 5 and 6). The association between apoSAA$_p$ and cholesterol is tight, K_d = 1.7 × 10^{-7} M, and the apoSAA$_p$-cholesterol complexes are eluted where dimers of apoSAA would be expected. PC altered the elution volume of apoSAA$_p$ only slightly (Fig. 6). Work is in progress to compare the kinetic parameters of phospholipid association with apoSAA to those reported here for cholesterol and apoSAA. As the apoA-I used in this study was purified from HDL, it may have contained some associated lipid. Future studies using recombinant apoA-I derived by expression of cloned apoA-I cDNA sequences may be expected to clarify this issue. The enhanced cholesterol flux from HDL to apoSAA$_p$ is not likely to be secondary to the formation of apoSAA$_p$-phospholipid complexes, as cholesterol exchange was about 4-fold greater on a molar basis than phospholipid exchange in the presence of apoSAA. The studies of Sparks et al. (29) have suggested a direct interaction of cholesterol with apoA-I even in the presence of excess phospholipid.

The chemoattractant activity of apoSAA$_p$ for monocytes and PMNs is inhibited by HDL (30). In view of the equimolar binding of cholesterol by apoSAA$_p$ and the high local concentrations of apoSAA during inflammation, we suggest that apoSAA may play a role in cholesterol transfer at local tissue sites after dissociation from HDL. Work is in progress to use double labeling techniques to investigate this issue, e.g., by formation of apoSAA-cholesterol complexes from incubation of [^{14}C]cholesterol with [^{3}H]SAA, [^{3}H]HDL lacking apoSAA, or [^{3}H]apoSAA-rich HDL.

We have demonstrated that [^{14}C]cholesterol uptake by HepG2 cells was increased by apoSAA$_p$ in a dose-dependent manner (Fig. 7). The enhancing effect of apoSAA$_p$ is blocked by antibodies to human plasma apoSAA (Table 3). ApoSAA$_p$ is taken up and degraded by a cell-associated mechanism by in the case of HepG2 cells, whereas peritoneal exudate cells can degrade apoSAA$_p$ extracellularly (Fig. 9). It is possible that apoSAA clearance may involve lysosomal enzymes such as elastase and cathepsins during the endocytic catabolism of HDL particles. The availability of recombinant preparations of apoSAA isoforms will enhance future studies of the hepatic clearance of apoSAA$_p$ isoforms.

In conclusion, our study has shown that the recombinant hybrid apoSAA$_p$ binds cholesterol and modulates cholesterol flux. The direct interaction of apoSAA$_p$ with cholesterol results in dimerization or in a change in conformation such that apoSAA is eluted at the position where a dimer would be expected. These previously undescribed properties of apoSAA could provide important clues as to how the normal amphipathic helical, HDL-associated, rapidly catabolized apoSAA is converted to insoluble amyloid A fibrils with the cross-β pleated sheet conformation.

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