Characterization of constitutive human serum amyloid A protein (SAA₄) as an apolipoprotein

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Abstract Serum amyloid A proteins (SAAs), a family of homologous molecules, are apolipoproteins of high density lipoprotein (HDL). They can be divided into two groups. The first group comprises the well-characterized acute phase SAAs that associate with HDL during inflammation, thereby remodeling the HDL particle by displacing apolipoprotein (apo)A-I. The second group consists of the recently discovered constitutive SAAs, mouse SAA₅ and human SAA₄. They exist as minor apolipoproteins on HDL but constitute more than 90% of the total SAA during homeostasis. We have characterized human SAA₄ as an apolipoprotein. During homeostasis, SAA₄ is synthesized only in the liver. Purification of SAA₄ has been described and its plasma concentration has been established at 55 ± 13 μg/ml in 26 healthy individuals. It was present on all HDL density classes and very low density lipoprotein (VLDL) but was absent from low density lipoprotein (LDL).

High density lipoprotein (HDL) plays a central role in lipid metabolism by continuously exchanging components with cells and other lipoproteins (1). HDL particles display a dynamic polydispersity with respect to size, hydrated density, and apolipoprotein composition (2). Apolipoproteins fulfill important biological roles by acting as ligands for receptors or co-factors for enzymes (3). Serum amyloid A proteins (SAAs), a family of homologous molecules, are all apolipoproteins of HDL (4, 5). They can be divided into two groups (6). The first group includes the well-characterized classical acute phase SAAs that increase dramatically during an acute phase response due to cytokine-driven hepatic synthesis (7). They displace apolipoprotein A-I (apoA-I) with resultant remodeling of HDL, yielding larger particles with a higher hydrated density (2), even becoming the major apolipoprotein component of acute phase HDL (8). The second group comprises the recently discovered constitutively expressed SAAs, namely mouse SAA₅ (9) and human SAA₄ (6). They exist as minor apolipoproteins of normal HDL comprising 1–2% of the total apolipoprotein component during homeostasis (6, 9).

Four human SAA genes are located on chromosome 11 (10, 11). The acute phase SAAs are encoded by two genes, SAA₁ and SAA₂. Allelic variation at these two loci accounts for the acute phase SAA isoforms identified to date (12, 13). The locus designated SAA₃ appears to be a pseudogene because of the presence of an extra base in exon 2 (14). This is corroborated by the fact that neither the protein product nor message for this gene has been identified. The SAA₄ locus identified recently encodes the constitutively expressed SAA₄ (10).

In this paper, we localized and characterized SAA₄ on HDL subpopulations, a prerequisite for future functional analyses. We define the synthesis site of SAA₄ and describe techniques for its purification. During the acute phase response, when the cytokine-inducible SAAs dominate on a molar basis, SAA₄ remains associated with HDL as a minor apolipoprotein. Using two-dimensional electrophoresis and phosphorimaging, SAA₄ is found to be associated with a specific subclass of HDL particles unrelated to those involved in the initial cholesterol transfer from cells (15).

Supplementary key words high density lipoprotein • acute phase

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; IEF, isoelectric focusing; SAA, serum amyloid A protein.

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MATERIALS AND METHODS

Preparation of lipoproteins

Lipoproteins were isolated by sequential ultracentrifugation from the blood of healthy donors or from patients experiencing an acute phase response after surgery (16). HDL was further subfractionated according to plasma density (d); HDL$_2$ (d 1.063-1.13 g/ml), HDL$_{3A}$ (d 1.13-1.155 g/ml), HDL$_{3B}$ (d 1.155-1.18 g/ml) and HDL$_{3C}$ (d > 1.18 g/ml) by recentrifugation of the total HDL fraction into a linear KBr gradient as described previously (2).

Purification of SAA$_4$

SAA$_4$ was isolated from HDL by Rotofor (Bio-Rad, Richmond, CA) preparative isoelectric focusing as per the manufacturer's instructions. Briefly, 200 mg of normal HDL was delipidated with ethanol–ether 3:2 (v/v) and the protein pellets were suspended in 15 ml 8 M urea, 1% (w/v) decyl sodium sulfate (Eastman Kodak Co., Rochester, NY) and 5% (v/v) 2-mercaptoethanol. The Rotofor running buffer consisted of 8 M urea, 1.2% ampholines pH 4-6.5, 1.2% ampholines pH 7-9, and 0.6% ampholines pH 3-10. The anionic and cationic chambers contained 0.1 M phosphoric acid and 0.1 M sodium hydroxide, respectively. The sample was electrofocused at constant power (12 W) until equilibrium was achieved. Twenty fractions were harvested and analyzed by SDS-PAGE. Those containing SAA$_4$ were pooled and subjected to molecular sieve chromatography to separate contaminating apolipoproteins. Briefly, the SAA$_4$-containing fractions were dialyzed against 15 mM NaCl, 2 mM Tris/HCl, pH 8.4, and lyophilized. The lyophilized pellet was suspended in 2 ml 7 M urea, 150 mM NaCl, 20 mM Tris/HCl, pH 8.4, and subjected to molecular sieve chromatography on a 1 × 120 cm Sephacryl S200 column as per the manufacturer's instructions (Pharmacia LKB Biotechnology, Piscataway, NJ) (17).

SAA$_4$ assay

SAA$_4$ was measured in plasma samples with an immunoradiometric method using rabbit anti-human SAA$_4$ antibody as described for inflammatory SAA$_4$s (18). A standard curve was obtained by using SAA$_4$-enriched HDL instead of purified SAA$_4$, that is relatively insoluble and aggregates in solution. This was prepared by incubating 1 mg purified SAA$_4$ with 1 mg normal HDL in 20 mM Tris/HCl, pH 7.4, 150 mM NaCl for 1 h at 22°C with gentle shaking. The HDL was separated from the free SAA$_4$ by ultracentrifugal flotation (2). Aliquots of this SAA$_4$-enriched HDL were subjected to SDS-PAGE using a 5-20% acrylamide gradient, and the Coomassie-stained SAA$_4$ bands were excised and quantitated by pyridine extraction of the dye as described (18).

Electrofocusing

Aliquots (50–400 µg) of lipoproteins were freeze-dried and delipidated with 0.5 ml chloroform–methanol 2:1 (v/v) (19). The delipidated proteins were suspended in sample buffer consisting of 8 M urea, 1% (w/v) decyl sodium sulfate (Eastman Kodak Co.) and 5% (v/v) 2-mercaptoethanol. Samples were electrofocused on 0.3-mm polyacrylamide gels containing 7 M urea and an ampholine gradient consisting of 20% (v/v) ampholines pH 3-10, 40% (v/v) ampholines pH 4-6.5 and 40% (v/v) ampholines pH 7-9 (Pharmacia-LKB Biotechnology) as described (16).

Immunochronal analysis

The SAA isoform distributions of lipoproteins were investigated by means of immunochronal analyses (16). Fifty-microgram samples of various lipoproteins were freeze-dried, delipidated, and subjected to isoelectric focusing as described above. Samples on electrofocused gels were pressure-blotted onto 0.2-µm pore-size nitrocellulose membranes (Schleicher and Schuell, Keene, NJ) for 20 h at room temperature. The membrane was wetted with 25 mM Tris/HCl, pH 8.3, 192 mM glycine, and 15% (v/v) methanol. After pressure-blotting, membrane binding sites were blocked overnight at 4°C with 5% (w/v) non-fat dry milk in PBS containing 2% (w/v) BSA. Screens for SAA isoforms were performed with a 1:1000 dilution of one of the following antibodies: our rabbit anti-human SAA$_4$ antibody (17), or a monoclonal anti-human SAA$_4$ antibody, or a monoclonal anti-human AA antibody. An alkaline phosphatase-conjugated goat anti-rabbit IgG antibody was used as secondary antibody (A8025, lot no. 39F-88961; Sigma Chemical Co., St. Louis, MO). The chromogenic substrates for alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt and nitroblue tetrazolium chloride (Bethesda Research Laboratories Life Technologies, Bethesda, MD) were applied according to the manufacturer's instructions.

Northern blot hybridization analysis

Human multiple tissue Northern blots containing poly A' RNA from a variety of human tissues, as well as peripheral blood leukocytes, (#7760-1 and 7759-1, Clontech Laboratories, Inc., Palo Alto, CA) were probed with our radiolabeled SAA$_4$ cDNA clone according to the manufacturer's instructions. Radiolabeled β-actin cDNA was used as a control probe. The blots were washed according to the manufacturer's instructions prior to exposure to film.

Two-dimensional separation of HDL particles

Two-dimensional separation of HDL particles was achieved as previously published (20). In the first dimen-
sion, plasma lipoproteins were separated by charge using agarose electrophoresis. In the second dimension, a separation by size was achieved using 3–36% non-denaturing concave gradient polyacrylamide gel electrophoresis.

Separated lipoproteins were transferred to a nitrocellulose membrane, localized by labeled monospecific antibodies, and quantitated by phosphorimaging (Phosphorimager SF, Molecular Dynamics, Sunnyvale, CA). Data were expressed as pixel points by computer analysis and were linearized with the dpm of the 125I-labeled antigen-antibody complexes. Radioactivity was integrated by the Molecular Dynamics Image Quant computer program. This method is capable of resolving several apoA-I-containing HDL subpopulations, including subclasses of α- and pre-α-migrating HDL, and a number of pre-β-migrating HDL subclasses. Particles were defined according to their relative $R_e$ to albumin (first dimension) and their size (second dimension).

### Immunoprecipitation of SAA4-containing HDL particles

SAA4-containing HDL particles were isolated from fresh human plasma by immunoabsorption. Briefly, 8 mg IgG fraction from rabbit anti-human SAA4 antisera was coupled via its carbohydrate moieties to Affi-gel HZ (Bio-Rad Laboratories) according to the manufacturer’s instructions. Fresh human plasma batches (1 ml in 9 ml PBS or 10 ml) were incubated, respectively, overnight with 3 ml immobilized IgG fraction at 4°C with gentle rotation. Bound SAA4-containing particles were eluted after extensive 4°C PBS washing with 7 M urea, 20 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, pH 8.4, and analyzed by SDS-PAGE using a 5–20% acrylamide gradient gel. In one experiment eluted SAA4-containing particles were dialyzed against 4 mM Tris/HCl, 30 mM NaCl, pH 8.4, followed by freeze-drying to one-fifth the original volume. These particles were re-applied to the immunoabsorber, eluted, and analyzed as above.

### RESULTS

#### SAA4 purification

In order to characterize SAA4 as an apolipoprotein, we developed a method to purify it from normal HDL by using preparative isoelectric focusing (preparative IEF) and subsequent molecular sieve chromatography. We isolated 200 mg of total HDL from 240 ml of normal human plasma (2). The final yield of SAA4 was 2.9 mg, which constitutes a 25% purification efficiency given the starting concentration of 51 pg/ml. Analytical IEF indicated that SAA4 isoforms have relatively basic pI values of 8.1, 7.9, and 7.3, while apolipoprotein C (apoC) and apolipoprotein A-II (apoA-II) have much more acidic pI values (pI < 6.0). Therefore, apoA-II and apoC that will co-chromatograph with SAA4 on molecular sieve chromatography can be separated clearly from SAA4 in the fractions eluted from the Rotofor apparatus. Figure 1 shows that when the fractions were analyzed by SDS-PAGE, apoC and apoA-II appeared in the acidic fractions (fractions number 2–5) (Fig. 1, top), while SAA4 appeared in the basic fractions (fractions number 13–19) (Fig. 1, bottom). The darkening at the bottom of the gel is the result of Amphilones. At this stage SAA4 was contaminated by apoA-I and ampholines that were removed by molecular sieve chromatography.

![Fig. 1. SDS-PAGE analysis of apolipoproteins separated by preparative isoelectric focusing. Normal human HDL (200 mg) was subjected to preparative isoelectric focusing as described in Materials and Methods, and aliquots of each of the eluted fractions were analyzed in a 5–20% acrylamide gradient reduced SDS gel. The Coomassie-stained gels depict the separation achieved for apoA-I, apoA-II, and SAA4. (ApoC is obscured by ampholines.) SAA4 eluting in fractions 13–19 show apoA-I contamination. S, normal human HDL (10 μg) loaded as a standard.](https://www.jlr.org/)

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Plasma concentration of SAA₄

Plasma SAA₄ concentrations were measured with an immunoradiometric assay similar to our method reported previously for inflammatory SAAs (18). Standardization was achieved by creating artificial SAA₄-enriched HDL particles as depicted in Figure 2, lane 2. (The apparent decrease in apoA-II results from gel “smiling.”) In 26 healthy volunteers, the plasma concentration of SAA₄ was 55 ± 13 μg/ml. Thus, the concentration was very comparable to that of the C apolipoproteins.

Tissue expression of SAA₄

Poly A⁺ RNA from 15 different human organs and peripheral blood leukocytes were hybridized to the radiolabeled SAA₄ cDNA clone CS1. Northern blot analyses showed SAA₄ expression during homeostasis only in liver tissue (Fig. 3). The size of the SAA₄ mRNA was similar to the approximately 700 bases reported previously (6). The Northern blot obtained from the first eight organs was subsequently hybridized to radiolabeled β-actin cDNA to verify the intactness of the RNA, showing the presence of β-actin in the organs analyzed, as well as α-actin in heart and muscle (Fig. 3).

Distribution of SAA₄ among lipoprotein classes and HDL subclasses

Immunochemical staining of isoelectric-focused LDL and VLDL obtained from normal individuals showed that SAA₄ was present also in VLDL but not in LDL.
In addition to the major SAA$_4$ isoforms (pI 7.3, 7.9, and 8.1), minor isoforms of pI 6.8, 6.25, and 6.20, confirmed to be SAA$_4$ by amino-terminal amino acid sequencing, are also evident in VLDL. These minor isoforms constitute approximately 5% of the total SAA$_4$ component. The same results were obtained with LDL and VLDL from patients in acute phase (data not shown). SDS-PAGE analyses of HDL particles with a wide range of densities (d 1.063–1.21 g/ml) showed that both the glycosylated and nonglycosylated molecules are present in similar ratios and amounts in HDL$_{2a}$, HDL$_{3a}$, and HDL$_{3b}$ (data not shown).

**SAA$_4$ is present on acute phase HDL**

SAA$_4$ is a minor apolipoprotein component of normal HDL constituting 1–2% of the total apolipoproteins of this particle. It was the dominant form of SAA on normal HDL, the acute SAAs being virtually undetectable (Fig. 5A). In patients mounting an acute phase response, SAA$_4$ was not displaced by the vastly increased number of inflammatory SAA molecules on HDL; its presence was masked by the dominance of the acute phase SAAs (Fig. 5B). We also analyzed the acute phase SAAs in patients undergoing an acute phase response. We observed that both SAA$_4$ and the acute phase SAAs co-localize to the same HDL subpopulations (unpublished observation, B. Asztalos, P. S. Roheim, and F. C. de Beer). The acute phase SAAs are encoded by two genes with allelic variation possible at each locus (12, 13). Each allele gives rise to two isoforms, the primary translation product and a post-translational modification, thus accounting for eight possible isoforms (12, 13). In the immunoblot presented in Fig. 5C, the acute SAA isoforms on the HDL of three patients were identified with a monoclonal anti-human AA antibody specific for acute phase SAA$_1$ and SAA$_2$. These patients had respective acute phase SAA concentrations of 167, 579, and 290 µg/ml. All three patients were homozygous at the SAA$_1$ gene locus. The protein product of this gene (SAA$_1$) was represented by the primary translation product (pI 6.4) and its post-translational modification (pI 6.0). Patients #1 and #3 were also homozygous at the SAA$_2$ gene locus. The protein encoded by this allele, SAA$_{2a}$, was represented by the primary translation product (pI 7.5) and its post-translational modification (pI 7.0). Patient #2, however, was heterozygous at the SAA$_2$ gene locus. Here SAA$_{2a}$ and SAA$_{2b}$ were represented by the respective primary translation products (pI 7.5 and 8.0) and their post-translational modifications (pI 7.0 and 7.4). Fig. 5B is a Coomassie stain of the HDL from the same three patients represented in Fig. 5C, showing the acute SAA isoforms evident in Fig. 5C, as well as the SAA$_4$ isoforms evident in Fig. 5A. In patients heterozygous at the SAA$_2$ gene locus (such as patient #2), the presence of the SAA$_4$ isoforms is masked particularly by the overexpression of the basic acute phase SAA$_{2b}$ isoforms. However, in patients who are homozygous at the SAA$_2$ gene locus, the constitutive SAA$_4$ can be readily distinguished from acute SAA isoforms by IEF (patient #1, patient #3). The amount of SAA$_4$ on the HDL particles during the acute phase is unaltered from that on normal HDL; SAA$_4$ is thus not appreciably displaced.

**SAA$_4$ is present on a particular subpopulation of HDL particles**

Ultracentrifugal separation of lipoproteins is not as sensitive a method of lipoprotein fractionation as two-dimensional electrophoresis. During ultracentrifugation, losses and/or incomplete separation of some functionally important particles may occur (15, 20).

We subjected fresh, normal plasma from three individuals to two-dimensional electrophoresis using 0.7% agarose in the first dimension to separate particles according to mobilities and non-denaturing polyacrylamide gradient gels in the second dimension to separate particles of different sizes. With the aid of a monospecific rabbit anti-human SAA$_4$, we determined a remarkably selective distribution of SAA$_4$ to three distinct particles (Fig. 6). Two
of these particles (1 and 2) had similar sizes, but different charges. Particle 3 was similar in charge to particle 2, but not in size (Table 1). From the first-dimensional distribution, (top insert, Fig. 6), it was apparent that there were two separate \( \alpha \)-migrating particles, which represented over 95% of SAA4. Presence of pre-\( \beta \)-migrating particles in the first dimension was indicated. This went undetected on two-dimensional electrophoresis. This probably represents VLDL-associated SAA4. It should be noted that the \( R_s \) of the SAA4-carrying particles were very similar in the three individuals studied, but the percent distribution of SAA4 between individuals varied somewhat (Table 1).

Characterization of the apolipoprotein component of SAA4-containing particles

Solid phase immunoadsorption using monospecific anti-SAA4 antibodies revealed that SAA4 was present on particles that had apoA-I, apoA-II and apoC present in ratios indistinguishable from those of normal HDL3 prepared by ultracentrifugation (data not shown). When small volumes of plasma (1 ml) were incubated with the solid phase, the composition of the eluted particles remained unaltered when compared to offering 10 ml of plasma. When eluted particles were re-constituted in a physiological buffer and re-applied to the immunoadsorbent, elution and analysis indicated that the apolipoprotein ratios were unaffected from the original (data not shown). This indicated it was unlikely that SAA4-enriched particles existed as reported for inflammatory SAAs during the acute phase (2).

**DISCUSSION**

Until now, the view was prevalent that SAA molecules were present on normal HDL in very insignificant amounts and that these same molecules increased dramatically during inflammatory events to become the major apolipoproteins on acute phase HDL. Our finding that there are two distinct groups of SAA links the function of the SAA family more intimately to that of HDL. Minor apolipoproteins can have major biological roles (3). The potential for SAA4 to play a similar role in norm-
nal HDL function merits consideration. Presumably the acute phase SAAs have a different but related function that equips the HDL particle for host defense during inflammatory states. A number of studies have suggested that the function of SAA is linked to the inflammatory process and that HDL is merely a carrier for this molecule (21–23). Teleologic considerations, however, suggest that SAA is involved in HDL metabolism per se. This has been supported by studies showing altered HDL binding to cells when SAA is present on the particles (24, 25) and the significant influence that SAA has on lecithin:cholesterol acyltransferase (LCAT) (26).

The discovery of the constitutive SAA group that comprised more than 90% of SAA on normal HDL (6, 9) linked the function of the SAA family more directly to that of normal HDL. The distribution of the constitutive SAA molecules that were restricted to HDL subclasses and VLDL was similar to that of apoC (27) and provides support for this contention. The concentration of human constitutive SAA4 was comparable to that of apoC (27).

The basic isoelectric points of the constitutive SAA4 isoforms probably contributed to the delay in recognizing the existence of this group on normal HDL. Only a single gene has been identified (28), and the isoforms are probably the result of differential glycosylation because all these isoforms were identical through nine cycles of aminoterminal sequencing (data not shown).

The constitutive SAAs of human and mouse have been found to be structurally similar to each other, but distinct from the inflammatory SAA group (6, 9). All inflammatory SAAs in all species studied were conserved between amino acids 33 and 44 (29). The constitutive SAA molecules are unique in having substitutions in this region, as well as the characteristic additional octapeptide insert (6, 9). This suggests a distinct function for this group. The induction of the constitutive SAA group also differed from that of the inflammatory group (6, 9). Human SAA4 was not induced by cytokines, whereas constitutive mouse SAA2 was only modestly induced (8). However, it has recently been shown that the presence of mouse SAA5 in HDL during the peak of the acute phase response was prevented either by translational interference or by displacement from the particle and rapid clearance (9). This suggests that mechanisms operate to ensure the domination of either of the SAA groups on HDL, but not both at the same time (9).

Of interest was the relatively high tyrosine content of the constitutive SAAs. The inserts, for instance, had a double tyrosine motif (6, 9). It merits consideration whether this might have functional implications given the recent data showing oxidative tyrosylation of HDL by peroxidase-enhanced cholesterol removal from cultured fibroblasts and macrophages (30).

The advent of two-dimensional separation of lipoproteins from fresh plasma, on the basis of charge in the first dimension and size in the second dimension (20), has allowed for a much greater definition of the polydispersity of HDL particles (15). Thus, apoA-I-containing particles were divided into 12 distinct groups (15). It is remarkable that SAA4 was associated with only three discreet, closely

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TABLE 1. Two-dimensional coordinates and percent distribution of SAA4

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<thead>
<tr>
<th>First Dimension (Median R,)</th>
<th>Second Dimension (nm)</th>
<th>% of Total</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0.80 ± 0.005</td>
<td>8.76 ± 0.18</td>
</tr>
<tr>
<td>2</td>
<td>0.95 ± 0.020</td>
<td>8.65 ± 0.13</td>
</tr>
<tr>
<td>3</td>
<td>0.97 ± 0.020</td>
<td>8.01 ± 0.10</td>
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Values represent the mean ± SD of three subjects determined three times. SD, standard deviation. Plasma samples were subjected to two-dimensional electrophoresis as described in Materials and Methods. Relative R, to albumin was obtained after immunolocalization with antialbumin. The size of the particles was determined by constructing vertical rectangles around the internal standards and the HDL particles (20) and quantitating the incorporated radioactivity as set out in Materials and Methods. Modal diameters were calculated using computer-generated internal curves (20). Using the coordinated R, and size, each area was delineated and the pixel volume and % of total immunoradioactivity were calculated (20).
related particles. These particles were distinct from the particles that were shown to be involved in the initial acceptance of cholesterol from cells (20). Thus it is unlikely that constitutive SAA₄ was involved in this process. Additionally, our immunoabsorption data indicated that the ratios of apolipoproteins on SAA₄-carrying particles did not differ from the ratios obtained when a total HDL₃ population of particles was prepared from this plasma. Given that phospholipids have recently been identified as important factors in imparting charge to HDL particles (31), it makes it likely that these particles carry a distinct phospholipid component different from other HDL particles. This could be of considerable interest given that phospholipid transfer between lipoprotein particles remains ill-defined even though of obvious importance (32).

We propose that the function of the SAA family is linked to that of HDL. Studies indicating that inflammatory SAA-bearing HDL increased binding to cells (24, 25) raises the question whether lipid flow between cells and HDL is altered by the presence of inflammatory SAA on these particles. Constitutive SAA₄, on the other hand, merits consideration as a factor that might be involved in lipid transfer between lipoprotein classes.

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