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Murine serum amyloid A_3 is a high density apolipoprotein and is secreted by macrophages

(injury/acute phase/extrahepatic/lipopolysaccharide)

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ABSTRACT **The serum amyloid A (SAA) proteins make up a multigene family of apolipoproteins associated with high density lipoproteins. They are of ancient origin; the finding of a highly homologous protein in mammals and ducks indicates that SAAs have been in existence for at least 300 million years. The interspecies similarity among the SAAs makes the mouse, in which they have been most thoroughly studied, a reasonable model to use for defining the function(s) of this family of proteins in humans. Originally it was observed that the SAA proteins were made in the liver and represented a set of proteins belonging to acute-phase reactants. SAA_3 is a unique member of the SAA multigene family in mice in that its mRNA is also expressed in extrahepatic tissues by a variety of cell types, mainly macrophages and adipocytes. To date, nothing has been reported regarding the fate or function of the SAA_3 translation product. To identify the SAA_3 protein, we developed SAA_3-specific antibodies by immunizing rabbits against a portion of SAA_3 protein synthesized in a bacterial fusion protein expression system. Electroimmunoblot analysis of serum and lipoprotein fractions of it showed SAA_3 to be associated with high density lipoproteins of mice treated with lipopolysaccharide. Furthermore, a continuous mouse macrophage cell line (J-774.1), when exposed to lipopolysaccharide, expressed SAA_3 mRNA in a dose-dependent manner and secreted SAA_3 protein. The expression and secretion of SAA_3 by macrophages stimulated with lipopolysaccharide suggest a role for this SAA in local responses to injury and inflammation.**

Serum amyloid A (SAA) is a family of highly homologous high density lipoprotein (HDL) apoproteins found in mammals and ducks (1–4). They are normally present in only trace amounts but are elevated up to 1000-fold and reach concentrations of 1 mg/ml during an acute-phase response (5, 6). In the mouse, four genes, SAA_1, SAA_2, SAA_3 (7–10), and SAA_5 (11), are actively transcribed, and the corresponding proteins for SAA genes 1, 2, and 5 are found in the circulating HDL (5, 11). The SAA_3 protein, however, has not been identified. After injection of mice with lipopolysaccharide (LPS), SAA mRNA is detectable in the liver as well as extrahepatic sites (12, 13). SAA_1 and SAA_2 mRNAs are expressed primarily in liver; low levels of each are found in kidney, and a low level of SAA_1 mRNA, but no SAA_2 mRNA, is found in the intestine (12, 14). SAA_3 mRNA, in contrast, is induced in many tissues and is expressed at various levels by several different cell types (12). Macrophages are a principal cell type of extrahepatic SAA_3 expression (12, 15, 34). LPS stimulates the accumulation of steady-state SAA_3 mRNA in peritoneal macrophages to levels equal to those in hepatocytes (12). The association of SAAs with HDL during an acute-phase reaction indicates a role for this family of proteins in lipid metabolism or transport during the host response to injury. As of yet, no direct evidence has emerged supporting this or any other role for the SAA protein family. Because SAA_3 mRNA is expressed in macrophages, SAA_3 should be secreted into the local interstitial spaces, where it may have a special role in the extrahepatic response to injury. In addition, although SAA_3 is an acute-phase reactant, it may be expressed by locally activated macrophages without the involvement of the systemic, acute-phase reaction. To help gain insight into the functions of SAA_3, we developed anti-SAA_3-specific antibodies for detection of the protein. With these antibodies, we identified SAA_3 protein in the circulation and found it associated with HDL. Furthermore, we established that the SAA_3 mRNA and protein are expressed after LPS treatment of a continuous mouse monocyte/macrophage cell line (J-774.1). These findings and the structural characteristics of SAA (16) suggest possible functions for mouse SAA_3 and, by implication, for SAAs of humans and other species.

MATERIALS AND METHODS

Antibodies. Rabbit antibodies to synthetic SAA_3 peptides corresponding to amino acid residues 2–10 and 69–78 were produced, but none recognized the SAA_3 protein. Furthermore, antibodies raised in rabbits to a staphylococcal protein A/SAA_3 fusion protein expressed in *Escherichia coli* (17) were of low titer and limited use. Hence, another SAA_3 fusion protein was engineered in a vector that provided a protease cleavage site in the polylinker, thereby enabling production of SAA_3 protein. An *Sma* I fragment of a mouse SAA_3 cDNA (18), containing sequences coding for the COOH-terminal 55 amino acids (residues 49–103) plus 180 nucleotides of 3′ untranslated sequence, was subcloned into *Sma* I-cleaved pGEX-3X (Pharmacia LKB). Recombinant colonies of transformed DH5α (Bethesda Research Laboratories) were selected, and plasmids containing the SAA_3 cDNA in the proper forward orientation were identified by restriction mapping. One, pGEX/SAA_3-49, was chosen for fusion protein expression, which was induced with isopropyl β-D-thiogalactopyranoside. The fusion protein, glutathione *S*-transferase (GST)/SAA_3-49, was isolated by affinity chromatography and proteolytically cleaved with factor Xa (Boehringer Mannheim) (19). After separation from GST and factor Xa by filtration through a Centricon 10 device (Amicon) (Fig. 1), the SAA_3 peptide was injected into several rabbits; antiserum from one rabbit was used for these studies. The specificity of the antiserum was determined by electroimmunoblotting (see *Results*).

Rabbit antiserum to the COOH-terminal decapeptide of human SAA (residues 95–104), prepared in the laboratory of A. R. Steinmetz (University of Marburg, Marburg, F.R.G.),

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Abbreviations: SAA, serum amyloid A; HDL, high density lipoprotein; LPS, lipopolysaccharide; GST, glutathione *S*-transferase; IEF, isoelectric focusing; AA, amyloid A.

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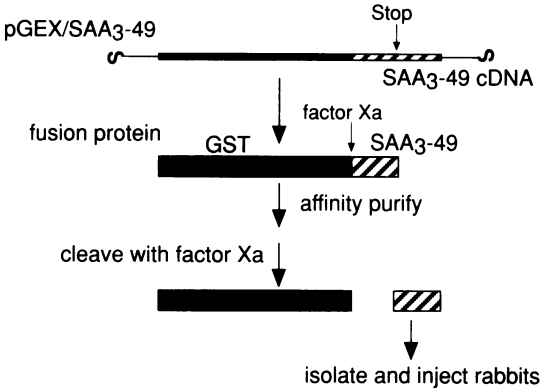

FIG. 1. Schematic illustration depicting ligation of SAA_3 cDNA into a GST gene fusion expression vector. A portion of an SAA_3 cDNA (hatched box), coding for the COOH-terminal 55 amino acids (residues 49–103) followed by ≈180 nucleotides of 3′ untranslated sequence, was ligated in-frame to the polylinker at the 3′ end of the GST coding sequence (solid box). Fusion protein (GST/SAA_3-49) expression was induced in recombinant *E. coli*, and the protein was affinity-purified from *E. coli* cell extracts on glutathione-agarose. GST/SAA_3-49 was eluted from the column and cleaved with factor Xa; the isolated SAA_3-49 peptide was injected into rabbits for antibody production.

was made available to us by F. C. de Beer. Antibodies to mouse amyloid A (AA) protein were raised in rabbits by injection (intradermal and intramuscular) of 50-μg doses of tissue-derived mouse AA (20) every 2 weeks.

Cell Culture. J-774.1 cells were grown in Dulbecco's modified Eagle medium (GIBCO) with 10% (vol/vol) fetal calf serum in a humidified incubator at 37°C in the presence of 10% CO_2. For assessment of their response to different doses of LPS, cells were grown to ≈95% confluence in 100-mm dishes, rinsed with phosphate-buffered saline, and incubated in 10 ml of serum-free medium with LPS at 0.1, 1.0, or 10 μg/ml or with no addition (control). After 16 hr the cells were harvested for RNA extraction. Medium to be tested for the presence of secreted SAA_3 protein was collected from control plates and plates incubated with LPS at 1.0 μg/ml, cleared of debris by centrifugation (10 min, ≈800 × *g*), and concentrated 300-fold in a Centricon 10 filtration device (Amicon). A time course study of SAA mRNA induction by LPS (1.0 μg/ml) was carried out with RNA extracted from cells harvested at various times after addition of LPS to the medium.

Northern Blotting. Samples of RNA (10 μg) extracted (21) from J-774.1 cells were electrophoresed through a denaturing 1.5% agarose gel, transferred to a nylon membrane, and hybridized with a random prime-labeled (22) SAA_3 cDNA as described (18).

Preparation of Serum Lipoprotein Fractions. Very low density, low density, and high density (HDL_2 and HDL_3) lipoprotein fractions were isolated by sequential density-increment centrifugation (23) of serum samples obtained from BALB/c mice 18 hr after intraperitoneal injection of 20 μg of LPS (*E. coli* 0111:B4; Difco) and from uninjected (control) mice. Lyophilized samples were prepared from delipidated aliquots of the dialyzed samples (23).

Isoelectric Focusing (IEF) and SDS/PAGE. IEF was done in a vertical slab gel apparatus with a chamber for circulating coolant (Hoefer SE 250 Mighty Small II). The gel formula was 7.5% acrylamide/0.2% *N,N′*-methylenebisacrylamide/8 M urea (ultrapure)/5.5% (vol/vol) Ampholines; the latter was a blend of three parts of pH 5–7, three parts of pH 7–9, and four parts of pH 9–11 preparations [40% (wt/vol); LKB or Sigma]. Gels were poured at room temperature; with a 10-tooth comb in place, polymerization was allowed to take place overnight at 4°C. The sample solvent was 5.5% (vol/vol) Ampholines/20% (wt/vol) glycerol/8 M urea/0.01 M H_3PO_4/200 μg of methyl green indicator per ml. For liquid samples, the solvent was augmented with Ampholines to 8%, glycerol to 30%, and H_3PO_4 to 0.02 M. The anolyte was 0.2 M urea/0.01 M H_3PO_4; the catholyte was 0.01 M NaOH. The polarity was such that positive ions moved downward in the gel. Coolant at 18°C was circulated through the cooling chamber. Focusing runs, preceded by a blank run with only solvent in the sample wells, were conducted at constant current (usually 3 mA per gel) for ≈1 hr. Under these conditions, chicken egg white lysozyme (14.3 kDa, pI 11) migrated to the lower edge of the gel or escaped it completely. Fixing and staining of gels were done according to Ordovas *et al.* (24), except that the staining, with Coomassie brilliant blue R-250, was done for 15 min at 55°C.

SDS/PAGE, done in precast gels (Jule, New Haven, CT) or in gels containing 7 M urea, was performed essentially as described (5).

Immunoblot Analysis. Samples were transferred from gels to nitrocellulose (BA85; Schleicher & Schuell) in a Hoefer TE22 Mighty Small Transphor apparatus overnight at 100 mA; the buffer was 25 mM Tris/192 mM glycine/0.01% SDS/10% (vol/vol) methanol. The electroblots were bathed for 1.5 hr in 1% bovine serum albumin/0.5% (vol/vol) polyoxyethylene sorbitan monolaurate (Tween 20)/0.15 M NaCl/0.05 M Tris/HCl, pH 7.7, to block unoccupied binding sites, for 2 hr in blocking buffer containing primary antiserum at a 1:1000 dilution, and for 0.5 hr in blocking buffer without bovine serum albumin (three changes) to remove unbound antibodies. The washed blots were bathed for 1 hr in blocking buffer containing alkaline phosphatase-conjugated goat anti-rabbit IgG (Fc), supplied at a concentration of 1 mg/ml (Promega) and used at a 1:10,000 dilution; unbound secondary antibodies were removed by washing, as described above. Color development was done in 0.1 M NaCl/5 mM $MgCl_2$/0.1 M Tris/HCl, pH 9.5 (filtered if cloudy), containing, per 10 ml, 40 μl of nitroblue tetrazolium [50 mg/ml in 70% (vol/vol) dimethylformamide] and 20 μl of 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in dimethylformamide); both of the latter were from Promega. The reaction was stopped by repeated rinsings with distilled water.

RESULTS

SAA_3 Circulates Associated with HDL. Serum samples from control, casein-injected, and LPS-injected mice were resolved by SDS/PAGE and examined by immunoblot analysis (Fig. 2*A*). An ≈12-kDa protein immunoreactive with anti-SAA_3 was identified in the serum of LPS-injected mice (lane 3) but not in that of control mice (lane 1) or casein-treated mice (lane 2). Lane 2 shows that anti-SAA_3 does not cross-react with SAA_1 or SAA_2, which are found in the serum of casein-treated mice; lane 4, containing serum from casein-treated mice, demonstrates the presence of SAA_1 and SAA_2 by immunoreactivity with anti-mouse AA, which recognizes SAA_1 and SAA_2. Immunoblot examination of HDL_3 isolated from serum revealed SAA_3 in the fraction from LPS-treated mice (lane 5) but not in that from control mice (lane 6); SAA_3 was detected in the HDL_2 fraction from serum of LPS-treated mice, but at a lower level than in the HDL_3 fraction, and not at all in the very low and low density lipoprotein fractions (data not shown). Because the expected pI of SAA_3 is predicted to be significantly more basic than the pI values of known mouse SAAs, delipidated proteins from the post-LPS HDL_3 fraction were resolved by nonequilibrium IEF, and immunoblots were probed with antibodies of different specificities (Fig. 2*B*). Anti-SAA_3 revealed a protein in the basic region of the pH gradient (lane 7), in a position slightly trailing that of chicken egg white lysozyme (pI 11), confirming the prediction of a very basic pI for SAA_3. Anti-human SAA (residues 95–104) identified SAA_5 (pI 8.1), SAA_1 (pI 6.35),

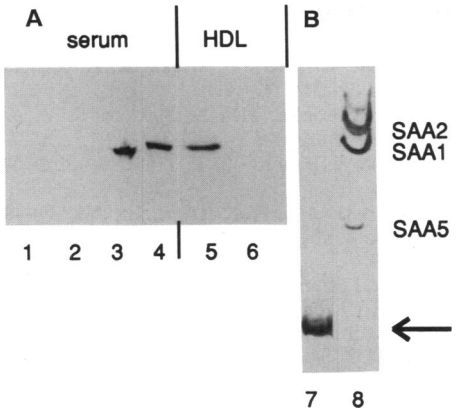

FIG. 2. Electroimmunoblot detection of SAA_3 in serum and on HDL. (*A*) Samples were resolved by electrophoresis on SDS/15% acrylamide gels. Lanes: 1, whole serum obtained from control mice; 2 and 4, whole serum from casein-injected (16 hr) mice; 3, whole serum from LPS-injected (18 hr) mice; 5, HDL_3 apoproteins isolated from serum of LPS-injected mice; 6, HDL_3 apoproteins isolated from serum of control mice. The primary antibodies used were anti-mouse SAA_3 (lanes 1, 2, 3, 5, and 6) and anti-mouse AA (lane 4). (*B*) Samples (20 μg) of delipidated HDL_3 apoproteins from LPS-treated mice were resolved by nonequilibrium IEF and probed with anti-mouse SAA_3 (lane 7) and anti-human SAA (residues 95-104) (lane 8). Isoelectric points reported for mouse SAA_2, SAA_1, and SAA_5 are, respectively, 6.20 (5), 6.35 (5), and 8.1 (11); the pI of mouse SAA_3 (arrow) was calculated to be 10.35 (25). All primary antibodies were used at a 1:1000 dilution.

and SAA_2 (pI 6.20) (lane 8). The amount of SAA_3 residing on HDL (18 hr after LPS treatment) is less than that of SAA_1 or SAA_2, as estimated from Coomassie blue staining of HDL proteins resolved by IEF (data not shown). The results presented in Fig. 2*B* also confirm the SAA_3 specificity of the anti-SAA_3 serum. Absorption of the anti-SAA_3 with GST/SAA_3-49 abolished its reactivity to HDL of LPS-treated mice (data not shown).

SAA_3 Protein Is Expressed in Macrophages. SAA_3 mRNA is expressed at high levels in hepatocytes and peritoneal macrophages in LPS-treated mice. We examined a mouse monocyte/macrophage cell line (J-774.1) to determine if SAA_3 mRNA was induced by LPS. J-774.1 cells were exposed to various amounts of LPS for 18 hr. Northern blot analysis of RNA hybridized with a ^{32}P-labeled SAA_3 cDNA revealed a dose-dependent induction of SAA_3 mRNA with a maximum at 1.0 μg of LPS per ml (Fig. 3*A*). J-774.1 cells have only a fraction (≈10%) of the SAA_3 mRNA found in liver RNA from an LPS-treated mouse (data not shown). A time course of SAA_3 mRNA induction by LPS (1.0 μg/ml) revealed that SAA_3 mRNA appeared between 2 and 4 hr after LPS exposure; it reached a maximum by 20 hr and decreased slightly by 24 hr (Fig. 3*B*).

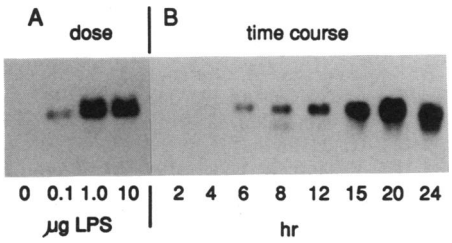

FIG. 3. Dose–response and time course of SAA_3 mRNA expression by mouse monocyte/macrophage cells exposed to LPS. RNA was isolated from J-774.1 cells 18 hr after exposure in medium without LPS or with LPS at concentrations of 0.1, 1.0, and 10 μg/ml (*A*) and after exposure for the indicated number of hours in medium with LPS at 1.0 μg/ml (*B*). Autoradiograms of RNA samples (10 μg) analyzed by Northern blot hybridization with a ^{32}P-labeled SAA_3 cDNA probe are shown.

To determine if SAA_3 protein was secreted by J-774.1 cells, culture medium collected from LPS-treated cells was concentrated and subjected to electroimmunoblot analyses (Fig. 4). A sample resolved by SDS/PAGE in a gel containing 7 M urea revealed an ≈12-kDa band reactive with anti-SAA_3 (lane 1); a sample resolved by nonequilibrium IEF (lane 2) revealed an anti-SAA_3-reactive band in a position closely matching that of the basic protein identified as SAA_3 in acute-phase mouse HDL_3 resolved by IEF. Nothing was detected by anti-SAA_3 in samples of concentrated medium from unstimulated J-774.1 cells (data not shown).

DISCUSSION

To our knowledge the protein encoded by murine SAA_3 mRNA has not been previously identified, although sequence data derived from cDNA indicated its existence (26). Our antibodies raised against a fragment of SAA_3 purified from a recombinant bacterial fusion protein permitted us to specifically identify the SAA_3 protein. We turned to an expression vector for antigen production after the failure of attempts to produce useful antibodies to peptide sequences of SAA_3 selected to provide specific epitopes. Construction of the expression vector was designed to exclude the highly conserved SAA sequence (residues 15–48 of SAA_3 show 90% and 82% sequence identity to SAA_1 and SAA_2, respectively) and to include the COOH-terminal 55 residues of SAA_3. In this region, SAA_3 differs from SAA_1 and from SAA_2 at 19 positions (65% identity), and the longest stretch of identity between SAA_3 and either SAA_1 or SAA_2 is 8 residues. With the specific anti-SAA_3 antibody, we identified SAA_3 in the circulation and found that it, like other SAAs, was associated with HDL. Furthermore, use of the antibody revealed the presence of SAA_3 among the proteins secreted by mouse monocyte/macrophages stimulated with LPS.

Previously we reported that extrahepatic SAA mRNA expression is restricted almost exclusively to that of SAA_3 (12), and by *in situ* hybridization we have identified cells expressing the specific mRNA. These cells include macrophages, adipocytes, and Leydig cells in addition to hepatocytes (15). Macrophages clearly contribute significantly to SAA_3 mRNA found in extrahepatic tissues. High levels of

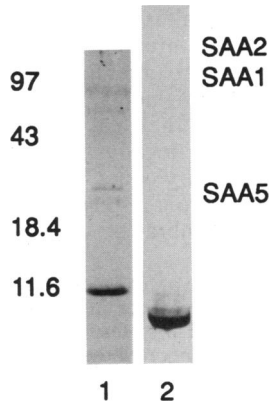

FIG. 4. Electroimmunoblot detection of SAA_3 in culture medium of mouse monocyte/macrophage cells. Medium collected 16 hr after exposure of J-774.1 cells to LPS (1.0 μg/ml) was concentrated 300-fold. Samples (6 μl) resolved by urea/SDS-PAGE (lane 1) and nonequilibrium IEF (lane 2) were probed with anti-mouse SAA_3. Molecular masses of reference proteins are indicated at the left (from top to bottom: rabbit muscle phosphorylase b, chicken ovalbumin, bovine β-lactoglobulin, duck AA), and nonequilibrium IEF positions of known mouse SAAs are indicated at the right.

SAA_3 mRNA, equal to those found in hepatocytes, are found in peritoneal macrophages (12), and SAA_3 mRNA is expressed in circulating macrophages and in resident splenic macrophages as seen by *in situ* hybridization (15). In the present work, we found that SAA_3 mRNA is expressed and its protein product is secreted by LPS-stimulated macrophages. This finding is compatible with the idea that SAA_3 plays a significant role in extrahepatic local injury responses. Moreover, SAA expression may occur locally apart from the general acute-phase reaction.

The SAA family of proteins shares an amphipathic helical structural motif with a number of other proteins and peptides having various biological functions. A principal feature of all of these proteins is their lipid-associating character. Among the proteins exhibiting this function are the apolipoproteins, such as apolipoprotein A-I, the C apolipoproteins, and apolipoprotein E, all of which are plasma constituents and exchangeable to a varying extent among the different lipoprotein classes. The prevailing assumption has been that the SAA proteins are made only in the liver and reach other locations via the circulation. However, as we have shown, SAA_3 is made by cells other than hepatocytes; although this isoform may contribute to the circulating pool of SAAs, it seems likely that it has a function locally where it is produced. Some potential functions are suggested in the following considerations. One effect of increased levels of SAA production is displacement of apolipoprotein A-I from the HDL particle (27). This is likely to alter interaction of HDL with cells since apolipoprotein A-I appears to be the major HDL ligand for several cell types (28–32). In support of this idea, unpublished studies in collaboration with J. F. Oram indicate that binding of SAA-rich mouse HDL to mouse monocyte/macrophages (J-774.1 cells) is significantly less than that of control HDL. Also of interest is the fact that macrophage production of apolipoprotein E, another HDL (and low density lipoprotein) apoprotein that promotes binding of lipoprotein particles to cell surface apolipoprotein B/apolipoprotein E receptors, is down-regulated by LPS (33). Expression of SAA_3 by macrophages at sites of injury may retarget the HDL not only by displacing apolipoprotein A-I but also by substituting for apolipoprotein E on HDL. Such retargeting of transported lipids, including cholesterol, or lipid-soluble toxins could function in disposal of toxic substances as well as delivery of cholesterol to alternative sites. Thus, the presence of SAA during local as well as general reaction to injury could alter the subset of cells that bind HDL. Furthermore, in interstitial spaces, macrophage-secreted SAA_3 could bind to other lipid substances, including cell membranes or their fragments, rather than to HDL, and function differently from the SAA_3 HDL apoproteins. Finally, in the case of the locally acquired SAA, the lipid particles in an area of injury could be routed to special sites for disposal or reuse by proliferating or regenerating cells for synthesis of new cell membranes.

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