

Peptides derived from serum amyloid A prevent, and reverse, aortic lipid lesions in apoE^{-/-} mice

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Abstract Macrophages (M ϕ) at sites of acute tissue injury accumulate and export cholesterol quickly. This metabolic activity is likely dependent on the physiological function of a major acute-phase protein, serum amyloid A 2.1 (SAA2.1), that is synthesized by hepatocytes as part of a systemic response to acute injury. Our previous studies using cholesterol-laden J774 mouse M ϕ showed that an N-terminal domain of SAA2.1 inhibits acyl-CoA:cholesterol acyltransferase activity, and a C-terminal domain enhances cholesteryl ester hydrolase activity. The net effect of this enzymatic regulation is to drive intracellular cholesterol to its unesterified state, the form readily exportable to an extracellular acceptor such as HDL. Here, we demonstrate that these domains from mouse SAA2.1, when delivered in liposomal formulation, are effective at preventing and reversing aortic lipid lesions in apolipoprotein E-deficient mice maintained on high-fat diets. Furthermore, mouse SAA peptides, in liposomal formulation, are effective at regulating cholesterol efflux in THP-1 human M ϕ , and homologous domains from human SAA are effective in mouse J774 cells. These peptides operate at the level of the foam cell in the reverse cholesterol pathway and therefore may be used in conjunction with other agents that act more distally in this process. Such human peptides, or small molecule mimics of their structure, may prove to be potent antiatherogenic agents in humans.—Tam, S. P., J. B. Ancsin, R. Tan, and R. Kisilevsky. Peptides derived from serum amyloid A prevent, and reverse, aortic lipid lesions in apoE^{-/-} mice. *J. Lipid Res.* 2005. 46: 2091–2101.

Supplementary key words atherosclerosis • cholesterol • acute-phase proteins • apolipoprotein E-deficient mice • high density lipoprotein

Acute tissue injury commonly results in local cell death and the generation of large quantities of cell membrane fragments rich in cholesterol. As part of the reactive acute inflammatory process, macrophages (M ϕ) are mobilized to such sites of injury, ingest these fragments, and acquire a considerable cholesterol load. A removal mechanism is required to mobilize this cholesterol either for reuse or

excretion. Our past results suggested that a physiological role of one isoform of a major acute-phase protein synthesized by the liver in response to tissue injury, mouse serum amyloid A 2.1 (mSAA2.1), is the regulation of M ϕ cholesterol export (1–3). Fragmentation of this protein into peptides that span its entire length has revealed that the N-terminal region, mSAA2.1_{1–20}, is a potent in vitro and in vivo inhibitor of M ϕ ACAT (3). A separate region at the C terminus, mSAA2.1_{74–103}, contains a domain that enhances the in vitro and in vivo activity of neutral cholesteryl ester hydrolase (CEH) (3). In combination, these two peptides drive stored cholesteryl esters into their unesterified form, which, in the presence of a functional cholesterol transporter and an extracellular cholesterol acceptor such as HDL, is rapidly exported from the M ϕ (2). These results suggested that such peptides may be useful in mobilizing cholesterol from M ϕ at sites of atherogenesis. To examine this possibility, we prepared liposomal formulations of the active SAA2.1 peptides and tested their ability to prevent, or cause the regression of, aortic lipid lesions in apolipoprotein E-deficient (apoE^{-/-}) mice maintained on a high-fat atherogenic diet. To determine whether these peptides, as liposomal formulations, are effective only in mouse M ϕ , we examined the influence of these peptides on 1) ACAT, CEH, and cholesterol efflux activities in cholesterol-laden THP-1 human M ϕ , and 2) the effect of homologous human peptides on cholesterol-laden J774 mouse M ϕ .

MATERIALS AND METHODS

Chemicals

All chemicals were reagent grade and purchased from Fisher Scientific (Nepean, Ontario, Canada), Sigma (St. Louis, MO), ICN (Aurora, OH), or Bio-Rad (Hercules, CA). DMEM, RPMI-1640

Abbreviations: AP-HDL, acute-phase high density lipoprotein; apoE^{-/-}, apolipoprotein E-deficient; CEH, cholesteryl ester hydrolase; M ϕ , macrophages; RBC, red blood cell; SAA, serum amyloid A.

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medium, and FBS were purchased from Life Technologies (Burlington, Ontario, Canada). Radiolabeled [^{14}C]oleic acid (52 mCi/mmol), [$^{1,2,6,7-3}\text{H}$ (N)]cholesterol (82 Ci/mmol), and cholesteryl- $^{1,2,6,7-3}\text{H}$ (N)oleate (84 Ci/mmol) were obtained from DuPont-New England Nuclear (Boston, MA).

Animals and diets

Swiss-white CD1 6–8 week old female mice were obtained from Charles River Laboratories (Montreal, Quebec, Canada). Mice were kept in a temperature-controlled room on a 12 h light/dark cycle. They were fed Purina Lab Chow pellets and water ad libitum. Female homozygous apoE $^{-/-}$ mice (C57BL/6J-*ApoE*^{tm1Unc}) were purchased from Jackson Laboratories (Bar Harbor, ME) at 8 weeks of age and fed Purina Lab Chow ad libitum until 12 weeks of age. For “prevention” experiments, apoE $^{-/-}$ mice were divided into six groups of animals. One group remained on lab chow and the other five were fed a high-fat diet containing 7.5% cocoa butter, 1.25% cholesterol, and 0.5% cholic acid (C13002; Research Diets, Inc.). When the animals were placed on the high-fat diet, they were given either no treatment or liposomes containing 15 μg of mSAA2.1 $_{1-20}$, 15 μg of mSAA2.1 $_{74-103}$, or a combination of both peptides (7.5 μg of each). Each liposome regimen was given every 4th day as a 100 μl intravenous injection via the tail vein. In addition, as a control, one group was given four injections of protein-free liposomes. For the “regression” experiment, mice were divided into five groups of animals. One group received the standard diet and the other four groups were fed the high-fat diet until they were euthanized. At 16 weeks of age, among the groups of mice receiving the high-fat diet for 4 weeks, one group received no treatment; the other three groups were given four injections of liposomes containing 15 μg of mSAA2.1 $_{1-20}$, 15 μg of mSAA2.1 $_{74-103}$, or a combination of these two peptides (7.5 μg of each). All animals were euthanized 4 days after the final injection of liposomes. Each of these experiments was performed twice.

En face quantitation of aortic lipid lesions

Animals were euthanized by CO $_2$ narcosis and exsanguinated by cardiac puncture into heparin-coated syringes for plasma lipid analyses. Total plasma, LDL, and HDL cholesterol and triglyceride levels were determined with Roche modular automated instruments in the clinical laboratories of Kingston General Hospital. The aortas were perfused with 10 ml of PBS via the left ventricle and teased free from the body but left attached to the heart. The adventitial adipose tissue was removed, and the aortas were opened longitudinally, pinned out as described (4), washed with 60% isopropanol for 3 min, stained with Oil Red O (0.4% in 60% isopropanol) for 3 min, rinsed in 60% isopropanol for 3 min, and then fixed in 10% formalin for 2 min. Once fixed, the aortas were stored in 10% formalin until the lipid lesions were quantified. Quantification of the percentage of the aortic surface occupied by Oil Red O-positive lesions was performed with a program and apparatus from MCID M2 Imaging Research, Inc. (St. Catharines, Ontario, Canada) as described previously (5).

Peptides

Peptides corresponding to mSAA2.1 $_{1-20}$, mSAA2.1 $_{21-50}$, mSAA2.1 $_{51-80}$, mSAA2.1 $_{74-103}$, and human (h)SAA1.1/2.1 $_{1-23}$ (note that the sequence of the human isoforms is identical over the first 50 residues) were prepared as described previously (3). In addition, an R linked to the N terminus of mSAA1.1 $_{1-20}$ was synthesized. These peptides were obtained from the Protein Synthesis Laboratory at Queen's University or The Hospital for Sick Children (Toronto, Ontario, Canada). The purity of the synthetic peptides was established by analytical HPLC and ion-spray mass spectrometry.

Preparation of red blood cell membranes as a source of cholesterol

To mimic the ingestion of cell membrane fragments by M ϕ at sites of tissue injury, red blood cells (RBCs) were used as a source of cholesterol, as described previously (2). Similar quantities of cholesterol, 175 μg , were used in all experiments. The concentration of cholesterol in the membrane preparations was determined as described previously (2).

Preparation of HDL and acute-phase HDL, and purification of apoA-I and SAA isoforms

HDL and acute-phase high density lipoprotein (AP-HDL) were isolated from normal and inflamed mice, respectively, using sequential density flotation as described previously (6, 7). The isolation, separation, and purification of apoA-I, SAA1.1, and SAA2.1 from acute-phase mouse plasma was performed as described previously (6, 7). The purity of the isolated proteins was established by mass spectrometry and N-terminal sequencing.

Preparation and characterization of apolipoprotein-lipid complexes (liposomes)

Each of the intact proteins (apoA-I, SAA1.1, and SAA2.1) or the various synthetic peptides listed above were reconstituted with lipids to form liposomes using the procedure described by Jonas, Kezdy, and Wald (8), as detailed previously (2, 3). When assessing the effects of the various apolipoproteins or peptides, these were always used as liposomes. Free peptides have no effect in culture or in vivo.

Cell culture

Mouse J774 M ϕ (TIB-67) and human THP-1 monocytes (TIB-202) were obtained from the American Type Culture Collection (Manassas, VA). J774 cells were cultured on six-well tissue culture plates at 10^6 cells/well and grown to 90% confluence in 2 ml of DMEM supplemented with 10% FBS. The medium was changed three times per week. THP-1 cells were maintained in RPMI-1640 medium containing 10% FBS according to the instructions supplied by the American Type Culture Collection. These monocytes were differentiated into M ϕ with 100 nM phorbol myristate acetate. The cells were seeded onto six-well tissue culture dishes at 10^6 cells per well and maintained in medium containing phorbol myristate acetate (100 nM). Media were replaced every 2 days, and experiments were started after 7 days in culture, when the cells morphologically were M ϕ .

Cholesterol loading and determination of ACAT activity

THP-1 or J774 cells were loaded with cholesterol using RBC membrane fragments as described previously (2, 3). ACAT activity was determined by measuring the incorporation of [^{14}C]oleic acid into cholesteryl esters as described previously (2, 3). ACAT activity was determined in phorbol myristate acetate-treated THP-1 cells without cholesterol loading, in cholesterol-laden cells, and in cholesterol-laden cells that were then cultured in medium supplemented with 50 $\mu\text{g}/\text{ml}$ native HDL, AP-HDL, protein-free liposomes, or liposomes containing 2 μM (final culture concentration) murine apoA-I, mSAA1.1, or mSAA2.1. To map the domains in mSAA2.1 that decreased ACAT activity, THP-1 M ϕ were loaded with cholesterol and labeled as described above, then incubated with liposomes containing 7.5 μg of the peptide of interest. These included mSAA2.1 $_{1-20}$, mSAA2.1 $_{21-50}$, mSAA2.1 $_{51-80}$, and mSAA2.1 $_{74-103}$. To determine whether homologous human peptides had an effect on ACAT activity similar to mSAA2.1 $_{1-20}$ in J774 cells, these cells were loaded with cholesterol and labeled as described above, then incubated with liposomes containing hSAA1.1/2.1 $_{1-23}$, or mSAA1.1 $_{1-20}$ as a negative control, or mSAA1.1 $_{1-20}$ with R added at the N terminus. After 3 h

incubations in the media described above, the cells were incubated for 3 h with [^{14}C]oleate (9, 10), chilled on ice, and washed twice with PBS-BSA and twice with PBS and [^3H]cholesteryl oleate (6,000 dpm/well) added as an internal standard to monitor extraction efficiency. The lipids were analyzed by thin-layer chromatography as described previously (9, 10). The radioactivity in cholesteryl ester spots was corrected for the extraction efficiency and used as a measure of ACAT activity.

Rates of hydrolysis of cholesteryl ester in THP-1 cells

Rates of hydrolysis of radiolabeled cholesteryl ester in THP-1 cells were determined exactly as described previously with J774 cells in the presence of 2 $\mu\text{g}/\text{ml}$ Sandoz 58-035 (an ACAT inhibitor) to prevent the reesterification of liberated [^{14}C]oleate and free cholesterol (2, 3). At various times under the different culture conditions, cellular lipids were extracted and analyzed for cholesteryl ester radioactivity as described above.

Cholesterol efflux in tissue culture and in vivo

THP-1 and J774 cells were laden with cholesterol by incubating with RBC membrane fragments that had been equilibrated previously with 0.5 $\mu\text{Ci}/\text{ml}$ [^3H]cholesterol (2, 3). Cholesterol pools were allowed to equilibrate for 18 h in culture, and efflux was examined after treatment with the different isoforms of SAA, or their peptides, as described previously (2, 3). To determine cholesterol export in vivo, experiments were conducted as described and validated previously (2). Briefly, J774 M ϕ were laden with RBC membranes and [^3H]cholesterol as described above, washed with PBS/BSA, and then detached from the culture dishes. Five million cells in 200 μl of DMEM were injected into mice via the tail vein. At various times thereafter, ~ 25 μl of blood was collected from the tail vein of each animal into heparinized capillary tubes, then centrifuged for 5 min in an Adams Autocrit Centrifuge to separate RBCs from plasma. Cholesterol efflux was determined by measuring the appearance of [^3H]cholesterol in plasma by scintillation spectrometry. To study whether the export of cholesterol from these injected J774 cells to plasma is influenced by mSAA2.1 $_{1-20}$, mSAA1.1 $_{1-20}$, or hSAA1.1/2.1 $_{1-23}$, 100 μl of liposomes containing 15 μg of one of these peptides was injected intravenously, and at various times after this injection, ~ 25 μl of blood was collected from the tail vein of each animal and the plasma was analyzed by scintillation spectrometry.

Protein determinations

Protein concentration was determined by the method of Lowry and coworkers (11), with the aid of a Bio-Rad protein assay kit.

RESULTS

Prevention of aortic lipid lesions in apoE $^{-/-}$ mice by ACAT-inhibiting and CEH-enhancing peptides of mouse SAA2.1

Previous results with mSAA2.1 $_{1-20}$ and mSAA2.1 $_{74-103}$ acting on mouse M ϕ ACAT and CEH activities, respectively, and on M ϕ cholesterol export in culture and in vivo suggested that these peptides may have antiatherogenic activity (2, 3). To assess this possibility, the effects of mSAA2.1 $_{1-20}$ and mSAA2.1 $_{74-103}$, individually and in combination, were examined in apoE $^{-/-}$ mice placed on a high-fat diet. Two protocols were used, a prevention mode, wherein the treatment with the requisite peptides began as the mice were placed on the high-fat diet, and a regression mode,

wherein the mice were on a high-fat diet for 28 days before treatment with the requisite peptides commenced.

In the prevention mode, six groups of mice were examined, those on 1) the high-fat diet; 2) standard lab chow (low-fat diet); 3) the high-fat diet given mSAA2.1 $_{1-20}$; 4) the high-fat diet given mSAA2.1 $_{74-103}$; 5) the high-fat diet given mSAA2.1 $_{1-20}$ + mSAA2.1 $_{74-103}$; and 6) the high-fat diet given peptide-free liposomes. In each case, the peptides were given as liposomes once every 4 days (four doses), at the termination of which the animals were euthanized and the aortas were stained with Oil Red O. The endothelial surface was examined en face, and the aortic area positive for lipid was expressed as a percentage of the total aortic area. These data were then normalized relative to the mean lipid-positive area in the untreated group fed the high-fat diet. These data are shown in Fig. 1.

Mice on the low-fat diet had $67 \pm 7\%$ less area occupied by lipid lesions relative to mice on the high-fat diet. Among the groups on the high-fat diet, those treated with protein-free liposomes or liposomes containing mSAA2.1 $_{1-20}$, mSAA2.1 $_{74-103}$, or mSAA2.1 $_{1-20}$ + mSAA2.1 $_{74-103}$ had, respectively, $14 \pm 19\%$, $45 \pm 8\%$, $41 \pm 13\%$, and $73 \pm 5\%$ less area occupied by lipid lesions relative to mice on the high-fat diet. The value with protein-free liposomes is not significantly different from that of the mice on the high-fat diet itself. The value with mSAA2.1 $_{1-20}$ + mSAA2.1 $_{74-103}$ is equivalent to that of mice on the low-fat diet. The *P* values for the groups of mice treated with the various peptides are <0.05 and are indicated in the legend to Fig. 1. These data indicate that peptides mSAA2.1 $_{74-103}$, mSAA2.1 $_{1-20}$, and particularly mSAA2.1 $_{1-20}$ + mSAA2.1 $_{74-103}$ are effective at inhibiting aortic lipid accumulation in apoE $^{-/-}$ mice.

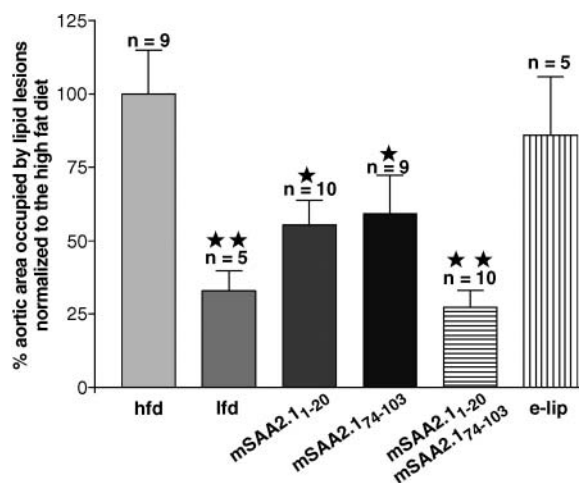


Fig. 1. The aortic area occupied by lipid lesions in animals on the prevention protocol (described in Materials and Methods) normalized to those in mice on the high-fat diet (hfd). e-lip, protein-free liposomes; lfd, low-fat diet; n, number of mice per group. mSAA2.1 $_{1-20}$, mSAA2.1 $_{74-103}$, and mSAA2.1 $_{1-20}$ + mSAA2.1 $_{74-103}$ indicate serum amyloid A (SAA) liposomes containing the designated peptide. The absolute area occupied by lipid lesions in the high-fat diet group is 1.05%. The values shown are means \pm SEM. One-way ANOVA was performed to determine statistical significance. One star, $P < 0.05$; two stars, $P < 0.01$.

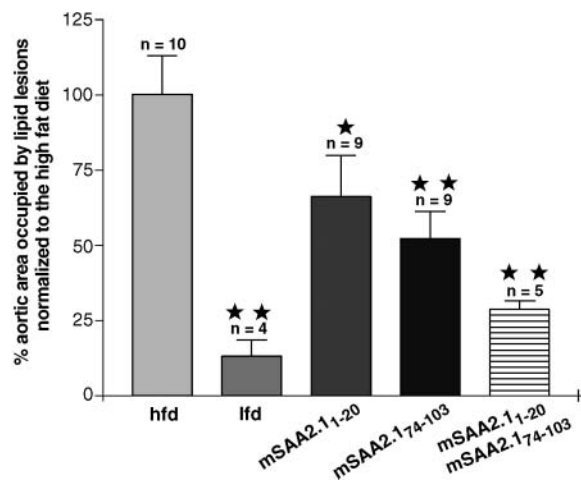


Fig. 2. The aortic area occupied by lipid lesions in animals on the regression protocol (described in Materials and Methods) normalized to those in mice on the high-fat diet. Abbreviations are as for Fig. 1. The absolute area occupied by lipid lesions in the high-fat diet group is 1.85%. The values shown are means \pm SEM. One-way ANOVA was performed to determine statistical significance. One star, $P < 0.05$; two stars, $P < 0.01$.

Regression of aortic lipid lesions in apoE^{-/-} mice by ACAT-inhibiting and CEH-enhancing peptides derived from mouse SAA2.1

In the regression mode, apoE^{-/-} mice were first placed on the high-fat diet for a period of 4 weeks. They were then divided into four groups, all of which continued on the diet for an additional 16 days; group 1 received no treatment, group 2 was treated with mSAA2.1₁₋₂₀, group 3 was treated with mSAA2.1₇₄₋₁₀₃, and group 4 was treated with mSAA2.1₁₋₂₀ + mSAA2.1₇₄₋₁₀₃. In each case, the peptides were administered as liposomes once every 4 days (four doses). A fifth group consisted of apoE^{-/-} mice on standard lab chow for 44 days. The results are illustrated in Fig. 2.

After 44 days, mice on the low-fat diet had $86 \pm 7\%$ less aortic area occupied by lipid lesions relative to those on the high-fat diet. Among the groups on the high-fat diet, those treated with mSAA2.1₁₋₂₀, mSAA2.1₇₄₋₁₀₃, or mSAA2.1₁₋₂₀ + mSAA2.1₇₄₋₁₀₃ had, respectively, $34 \pm 13\%$, $48 \pm 8\%$, and $71 \pm 4\%$ less area occupied by lipid lesions relative to those on the high-fat diet. The P values are indicated in the legend to Fig. 2. These data indicate that peptides mSAA2.1₇₄₋₁₀₃, mSAA2.1₁₋₂₀, and particularly mSAA2.1₁₋₂₀ + mSAA2.1₇₄₋₁₀₃ are effective at reducing aortic lipid accumulation in apoE^{-/-} mice. A visual comparison of the aortic lesions in an apoE^{-/-} mouse on a high-fat diet for 44 days and one treated with peptides mSAA2.1₁₋₂₀ + mSAA2.1₇₄₋₁₀₃ over the final 16 days is given in Fig. 3.

Effect of liposomes containing SAA peptides on plasma lipid parameters

The effect of the high-fat diet, and treatments, on plasma lipid parameters (triglycerides and total HDL and LDL cholesterol concentrations) are indicated in Table 1. There was a 3- to 5-fold increase in plasma cholesterol pa-

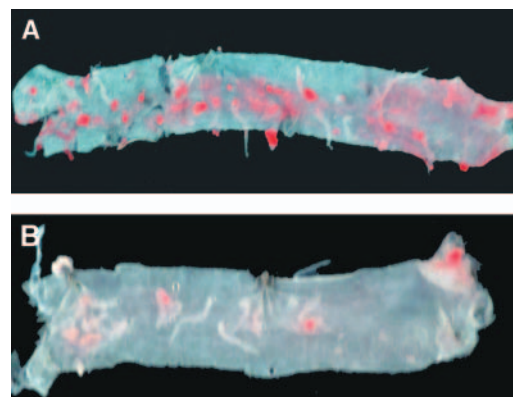


Fig. 3. A visual comparison of two aortas stained with Oil Red O viewed en face. A: Aorta from a mouse on the high-fat diet for 44 days. B: Aorta from a mouse on the high-fat diet for 44 days that for the final 16 days was treated every 4th day with mSAA2.1₁₋₂₀ + mSAA2.1₇₄₋₁₀₃, as described in Materials and Methods.

rameters when the mice were placed on the high-fat diet; however, there was no apparent effect of the peptides singly, or in combination, on these parameters, despite the fact that these peptides clearly affected the degree of the aortic lipid lesions. A comparison of the plasma lipid parameters between mice that were on the high-lipid diet for 16 days versus 44 days suggests a modest increase in HDL cholesterol in the latter group but a reduction in the other plasma lipid values in the groups fed this diet for longer periods of time. The reasons for these changes are not obvious but may relate to a reduction in the intake of diet over time (there was no difference in body weight between the mice in the various groups; data not shown), perhaps an increase in HDL production, or adaptations to a high-fat diet that are not immediately apparent. Nevertheless, it is important to note that the increase in HDL cholesterol and the decrease in LDL/HDL cholesterol ratio did not protect against the development of lipid lesions unless liposomes containing SAA peptides were also added.

Effect of mouse AP-HDL and liposomes containing SAA2.1 or SAA2.1 peptides on human THP-1 M ϕ

Our previous studies with J774 M ϕ demonstrated that mSAA2.1 had an ACAT-inhibitory domain at its N terminus and a CEH-enhancing domain at its C terminus (2, 3). Operating individually or in concert, these peptides have proven remarkably effective in culture and in vivo at promoting the rapid efflux of cholesterol from cholesterol-laden cells (2, 3). To determine whether these results are peculiar to mouse cells, we examined the effect of mouse AP-HDL, mSAA2.1, and mSAA2.1 peptides (each as liposomes) on ACAT and CEH activity and cholesterol export with a human M ϕ cell line, THP-1.

Figure 4 illustrates the baseline ACAT activity of THP-1 cells, the effect of feeding these cells mouse erythrocyte membrane fragments (as a source of cholesterol), and the subsequent influence of HDL or AP-HDL on such activity. As shown previously with J774 cells (2), loading the cells with cholesterol markedly increased THP-1 ACAT activity,

TABLE 1. Plasma lipid parameters of apolipoprotein E-deficient mice on standard lab chow, high-fat diet, and high-fat diet and treatment with mSAA2.1₇₄₋₁₀₃, mSAA2.1₁₋₂₀, or mSAA2.1₁₋₂₀ + mSAA2.1₇₄₋₁₀₃ in the prevention and regression protocols

Treatment	N	TG	TC	HDL-C	LDL-C	TC/HDL-C	LDL-C/HDL-C
LFD	5	1.00 ± 0.11	19.7 ± 2.30	8.90 ± 0.73	10.3 ± 1.60	2.16 ± 0.10	1.16 ± 0.10
HFD	9	3.01 ± 0.40	75.8 ± 6.41	20.8 ± 0.63	53.6 ± 6.00	3.62 ± 0.27	2.58 ± 0.26
mSAA2.1 ₁₋₂₀	10	3.07 ± 0.56	98.6 ± 4.80	25.6 ± 1.38	71.8 ± 4.65	3.97 ± 0.32	2.92 ± 0.31
mSAA2.1 ₇₄₋₁₀₃	10	3.49 ± 0.76	91.0 ± 6.30	23.4 ± 1.10	66.3 ± 5.30	3.92 ± 0.18	2.82 ± 0.17
Both peptides	10	3.61 ± 0.68	76.8 ± 7.50	19.9 ± 1.60	55.4 ± 6.80	3.93 ± 0.39	2.92 ± 0.38
PFL	5	2.00 ± 0.27	84.3 ± 3.40	23.0 ± 0.93	60.4 ± 2.80	3.54 ± 0.10	2.58 ± 0.12
*HFD	9	1.88 ± 0.27	74.1 ± 4.39	26.2 ± 2.20	47.1 ± 4.16	3.02 ± 0.33	1.97 ± 0.32
*mSAA2.1 ₁₋₂₀	9	1.69 ± 0.20	66.5 ± 4.55	25.0 ± 1.74	40.9 ± 3.22	2.71 ± 0.11	1.66 ± 0.09
*mSAA2.1 ₇₄₋₁₀₃	8	1.89 ± 0.23	72.5 ± 4.98	30.1 ± 3.66	41.6 ± 3.28	2.54 ± 0.20	1.53 ± 0.20
*Both peptides	6	1.12 ± 0.18	64.0 ± 2.11	26.9 ± 3.63	36.6 ± 1.92	2.40 ± 0.82	1.38 ± 0.07

Values, except for ratios, are in mmol/l ± SEM. N, number of mice per group; TG, total triglyceride; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; LFD, standard lab chow; HFD, high-fat diet; PFL, protein-free liposomes; mSAA, mouse serum amyloid A. Treatment groups with asterisks are from the regression protocol, as described in Materials and Methods.

which was not significantly reduced by subsequent exposure to HDL. However, subsequent exposure to AP-HDL resulted in a 60% reduction in the increased ACAT activity. Figure 4 also shows that this ACAT-inhibitory property of AP-HDL resides in mSAA2.1, analogous to that shown previously with J774 cells (2, 3). Furthermore, the domain responsible for the reduction in ACAT activity, as with J774 cells, resides in mSAA2.1₁₋₂₀ but not in peptides composed of mSAA2.1₂₁₋₅₀, mSAA2.1₅₁₋₈₀, or mSAA2.1₇₄₋₁₀₃.

The effects of AP-HDL, mSAA2.1, and peptides spanning the entire length of mSAA2.1 on CEH activity in

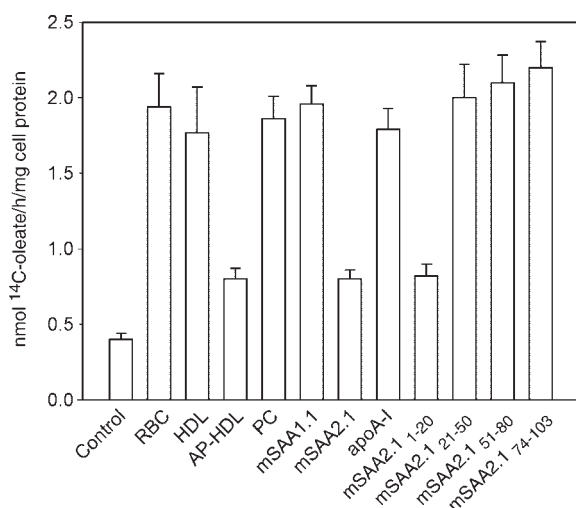


Fig. 4. Effects of cholesterol loading on human THP-1 macrophage (Mφ) ACAT activity and the subsequent effects of HDL, acute-phase high density lipoprotein (AP-HDL), or various protein- or synthetic peptide-containing liposomes on such activity. THP-1 Mφ were incubated in the absence (control) or presence of red blood cell (RBC) membrane fragments and labeled with [¹⁴C]oleate. ACAT activity was then determined as described in Materials and Methods with medium containing 0.2% BSA alone or the same medium plus protein-free liposomes (PC), with liposomes containing 2 μM (final culture concentration) murine apolipoprotein A-I (apoA-I), SAA1.1, or SAA2.1, or with liposomes containing 7.5 μg of synthetic peptides corresponding to amino acid residues 1–20, 21–50, 51–80, and 74–103 of mSAA2.1. The results shown are means ± SEM of four experiments.

THP-1 cells are shown in Fig. 5. Figure 5A illustrates the rate of breakdown of cholesterol oleate in the presence of medium alone, HDL, and AP-HDL. Only AP-HDL enhanced the esterase activity. Figure 5B shows that the enhanced esterase activity demonstrated in Fig. 5A was quantitatively attributable to mSAA2.1, because protein-free liposomes, and those containing apoA-I or mSAA1.1, had no such effect. Furthermore, the SAA2.1 domain responsible for the enhancement of CEH activity resides only in residues 74–103. These results are completely analogous to those seen previously with murine J774 cells (2, 3).

The effect of these peptides on cholesterol efflux from human THP-1 cells is illustrated in Fig. 6. These cells were laden with cholesterol and then pretreated for 4 h with HDL or the designated liposomes, washed free of the pretreatment, and placed into fresh medium containing HDL as the common cholesterol acceptor for the different pretreatments. At various times thereafter, the efflux medium was collected and the quantity of radiolabeled cholesterol released into the medium was determined. The response of the THP-1 cells was similar to that seen previously with J774 cells (2, 3). Those cells exposed to medium alone, or HDL alone, had the lowest rate of cholesterol efflux. Pretreatment with SAA2.1 peptides that correspond to the ACAT-inhibiting domain or the CEH-enhancing domain released cholesterol more rapidly and in greater quantity than the control treatments. Pretreatment with both peptides promoted the most rapid efflux of cholesterol, and in greatest quantity.

Comparison of mouse and human N-terminal domains on J774 Mφ ACAT activity, and cholesterol efflux in culture and in vivo

J774 mouse cells were laden with cholesterol and then exposed to medium alone, protein-free liposomes, or liposomes containing one of several mouse or human peptides corresponding to the N terminus of SAA2.1 or SAA1.1. As shown previously (2, 3) and in Fig. 4, loading Mφ with RBC membrane fragments increased their ACAT activity 5- to 6-fold. Subsequent exposure of these cells to protein-free liposomes had no effect on the increased ACAT

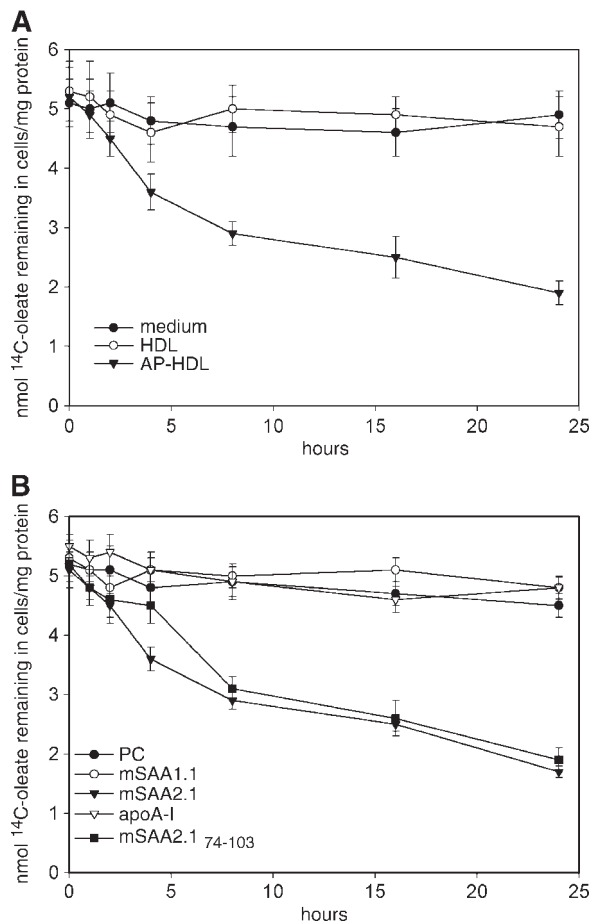


Fig. 5. Time course of cholesteryl ester hydrolase activity in cholesterol-loaded THP-1 M ϕ exposed to HDL, AP-HDL, or various liposomes. THP-1 M ϕ were cholesterol-loaded with RBC membrane fragments and labeled with [14 C]oleate. The cells were then incubated for up to 24 h in the presence of 2 μ g/ml of the ACAT inhibitor Sandoz 58-035 in 2 ml of medium containing 0.2% BSA alone or including 50 μ g/ml HDL or AP-HDL (A), in 2 ml of medium/BSA supplemented with protein-free liposomes (PC) or liposomes containing 2 μ M murine apoA-I, SAA1.1, or SAA2.1, or liposomes containing 7.5 μ g synthetic peptides corresponding to amino acid residues 74–103 of mSAA2.1 (B). After the indicated intervals, cellular lipids were extracted and analyzed for cholesteryl ester radioactivity. The results shown are means \pm SEM of four determinations.

activity (Fig. 4). Exposure of these cells to liposomes containing mSAA2.1₁₋₂₀ markedly reduced ACAT activity, an effect not shared by the homologous peptide corresponding to mSAA1.1₁₋₂₀ (Figs. 4, 7). Mouse SAA1.1₁₋₂₀ differs from mSAA2.1₁₋₂₀ by only two amino acids (see Discussion). In contrast, treatment of the cholesterol-laden M ϕ with liposomes containing hSAA1.1/2.1₁₋₂₃ (or hSAA1.1/2.1₁₋₂₀; data not shown) reduced the increased ACAT activity as well as mSAA2.1₁₋₂₀. As will be considered in Discussion, the result with hSAA1.1/2.1₁₋₂₃ (and hSAA1.1/2.1₁₋₂₀; data not shown) prompted us to examine the effect of mSAA1.1₁₋₂₀ with R added at the N terminus. R-mSAA1.1₁₋₂₀, in contrast to mSAA1.1₁₋₂₀, is a potent inhibitor of M ϕ ACAT activity (Fig. 7).

The effect on cholesterol efflux in J774 cells of the SAA peptides cited above is illustrated in Fig. 8. Cells were

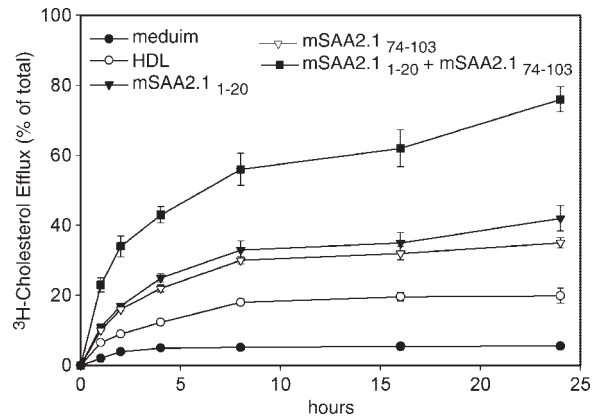


Fig. 6. Cholesterol efflux from cholesterol-loaded THP-1 M ϕ preincubated for 4 h with HDL or liposomes containing various synthetic SAA2.1 peptides, followed by 24 h with HDL. Cholesterol-loaded M ϕ labeled with [3 H]cholesterol were prepared as described for Fig. 1. Cells were preincubated in the absence or presence of either HDL (10 μ g/ml) or liposomes containing 7.5 μ g of synthetic peptides corresponding to amino acid residues 1–20, 21–50, 51–80, and 74–103 of mSAA2.1. In some experiments, the combination of mSAA2.1₁₋₂₀ + mSAA2.1₇₄₋₁₀₃ (7.5 μ g each) was also used. After incubation, the cells were washed extensively with medium/BSA to remove all radioactivity and various liposomes present in the preincubation medium. The chase efflux media consisted of medium/BSA alone or medium containing HDL (50 μ g/ml). The results indicated by the open circles represent cholesterol efflux to the acceptor, HDL, in the medium from cells without liposome pretreatment. At various times, the efflux media were collected and centrifuged at 10,000 g for 10 min, and [3 H]cholesterol in the supernatant was determined. Cellular lipids were analyzed for remaining free and esterified [3 H]cholesterol. The results are expressed as percentage of total (cell plus medium) radioactivity in each well. Total [3 H]cholesterol was $(1.3\text{--}1.5) \times 10^5$ dpm/mg cell protein. The results shown are means \pm SEM of four determinations.

laden with cholesterol and treated as described for Fig. 6. At various times thereafter, the quantity of radiolabeled cholesterol released into the medium was determined. It is apparent that mSAA1.1₁₋₂₀ was not effective at priming cholesterol export from the J774 cholesterol-laden cells. Pretreatment with this peptide did not enhance export above that seen when exposing the cells to the HDL acceptor alone. Recall, however, that mSAA1.1₁₋₂₀ had no effect on ACAT activity. In contrast, R-mSAA1.1₁₋₂₀, mSAA2.1₁₋₂₀, and hSAA1.1/2.1₁₋₂₃, the three peptides that inhibited ACAT activity, were equally effective at promoting cholesterol efflux to HDL in the medium. Similar comparisons have been made with mSAA2.1₇₄₋₁₀₃ and its human homolog and their effects on M ϕ CEH activity and cholesterol efflux, and similar results were obtained with both peptides (data not shown).

Figure 9 illustrates the effect of mSAA1.1₁₋₂₀, mSAA2.1₁₋₂₀, and hSAA1.1/2.1₁₋₂₃ on M ϕ cholesterol efflux in vivo. As in our previous work (2, 3), J774 cells were loaded with radiolabeled cholesterol in culture, washed extensively with medium, and then injected intravenously into recipient mice. Twenty-four hours later, the animals received a single intravenous injection of mSAA1.1₁₋₂₀, mSAA2.1₁₋₂₀, or hSAA1.1/2.1₁₋₂₃ as liposomes. Blood samples (25 μ L) were

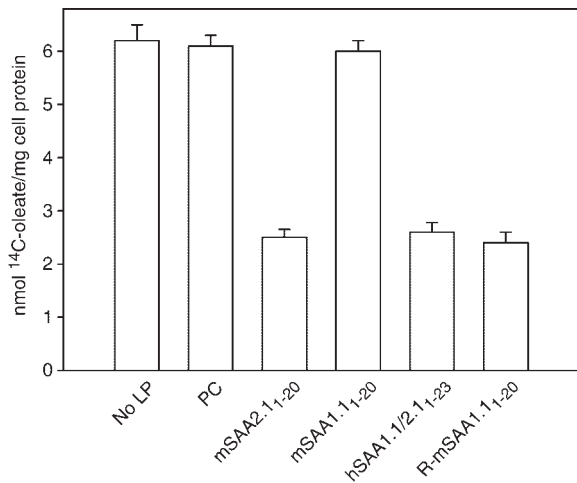


Fig. 7. Comparison of mouse and human N-terminal domains on murine J774 M ϕ ACAT activity. Nearly confluent monolayers of J774 cells were cholesterol-loaded and labeled with [³H]cholesterol as described in Materials and Methods. ACAT activity was determined in cholesterol-laden cells that had been cultured in DMEM/BSA alone (No LP), protein-free liposomes (PC), or liposomes containing 7.5 μ g of synthetic peptides corresponding to mSAA2.1₁₋₂₀, mSAA1.1₁₋₂₀, R-mSAA1.1₁₋₂₀, or hSAA1.1/2.1₁₋₂₃. The results shown are means \pm SEM of four experiments.

taken from the tail 12 h after the injection of the cells and at multiple times thereafter until 120 h. The quantity of isotope per microliter of plasma decreased steadily over 120 h in animals that did not receive an active peptide (2, 3), exemplified in Fig. 9 by mSAA1.1₁₋₂₀. Within 6 h of injection, mSAA2.1₁₋₂₀ or hSAA1.1/2.1₁₋₂₃ prompted a substantial release of cholesterol from the previously injected J774 cells, an effect that lasted at least 96 h, indicating that in mice the human N-terminal peptide is as active as its mouse equivalent.

DISCUSSION

SAA is a family of proteins encoded by four related genes whose evolutionary history is at least 600×10^6 years (12, 13). Of the four, isoforms 1.1 and 2.1 are major acute-phase proteins synthesized by the liver (14), and their plasma concentration may increase by two to three orders of magnitude after tissue injury, regardless of cause. Within 18–24 h of an inflammatory stimulus, 2.5% of total liver protein synthesis can be devoted to the production of these proteins (15), resulting in a dramatic increase in SAA's plasma concentration (from 1 μ g/ml to 1 mg/ml, a 500- to 1,000-fold increase). In plasma, 90% of SAA is found in the HDL fraction (16). In tissue culture, the acute-phase forms of SAA associate with HDL, displace apoA-I, and significantly remodel HDL's apolipoprotein composition (17, 18). Whether such a displacement also occurs in vivo has yet to be resolved (19). Nevertheless, during tissue injury, 30–80% of the apolipoprotein composition of AP-HDL is made up of the acute-phase isoforms of SAA in roughly equal proportions (20). HDL containing SAA (HDL/SAA) has a higher affinity for M ϕ

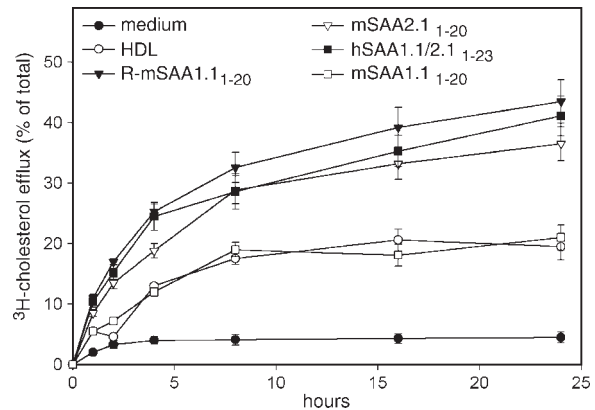


Fig. 8. Cholesterol export from cholesterol-loaded J774 M ϕ preincubated for 4 h with HDL, or liposomes containing various synthetic SAA2.1 peptides, followed by 24 h with HDL. J774 cells were cholesterol-loaded and labeled with [³H]cholesterol as described in Materials and Methods. Cells were preincubated in the absence or presence of HDL (10 μ g/ml) or liposomes containing 7.5 μ g of synthetic peptides corresponding to mSAA2.1₁₋₂₀, mSAA1.1₁₋₂₀, R-mSAA1.1₁₋₂₀, or hSAA1.1/2.1₁₋₂₃. After incubation, the cells were washed extensively with medium/BSA to remove all radioactivity and various liposomes in the preincubation medium. The chase efflux media consisted of medium/BSA alone or medium containing HDL (50 μ g/ml). The results indicated by the open circles represent cholesterol efflux to the acceptor, HDL, in the medium from cells without any liposome pretreatment. At various times, the efflux media were collected and centrifuged at 10,000 g for 10 min., and [³H]cholesterol in the supernatant was determined. Cellular lipids were analyzed for remaining free and esterified [³H]cholesterol. The results are expressed as percentage of total (cell plus medium) radioactivity in each well. Total [³H]cholesterol was $(2.5\text{--}2.7) \times 10^5$ dpm/mg cell protein. The results shown are means \pm SEM of four determinations.

(21), and M ϕ from inflamed animals have a greater number of binding sites for HDL/SAA (21). Such SAA is readily taken into the M ϕ (22, 23), and, as shown previously, the 2.1 isoform inhibits ACAT and enhances CEH activities, promoting the rapid export of cholesterol from such cells (2, 3). This property of mSAA2.1 has also been demonstrated in vivo (2, 3). M ϕ laden with cholesterol and radiolabeled cholesterol, when injected intravenously into mice and allowed to establish themselves for 24 h, rapidly and readily release their cholesterol to plasma only when prompted to do so with liposomes containing mSAA2.1 but not other apolipoproteins, such as mSAA1.1 or apoA-I (2, 3). This effect requires a functioning ABCA1 transporter, which indicates that the injected M ϕ remain viable during this in vivo experiment (2). These culture and in vivo properties of mSAA2.1 have been shown to reside in two domains. The ACAT-inhibiting domain is at the N terminus, and the CEH-enhancing domain is at the C terminus. Intervening peptides between residues 20 and 74 fail to influence ACAT and CEH activities and cholesterol efflux in cultures or in vivo (3). We have now demonstrated that these findings are not peculiar to mouse SAA and mouse M ϕ . Mouse peptides are equally effective with a human M ϕ cell line, and the homologous domains in human SAA are effective in the J774 mouse M ϕ cell line.

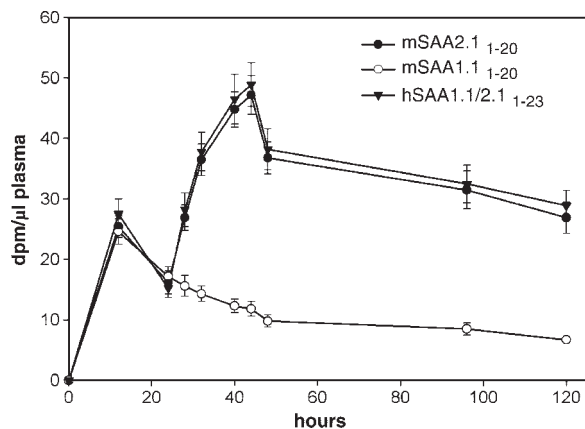


Fig. 9. Cholesterol export studies in vivo. J774 cells were cholesterol-loaded and labeled with [³H]cholesterol as described in Materials and Methods. Cells were then washed extensively and detached from the culture dishes. Five million cells in 200 μl of DMEM were injected intravenously into mice through the tail vein. After 24 h of equilibration, the animals were injected with 100 μl of liposomes containing 15 μg of synthetic peptides corresponding to mSAA2.1₁₋₂₀, mSAA1.1₁₋₂₀, or hSAA1.1/2.1₁₋₂₃. Cholesterol export was determined over a 96 h period by measuring the appearance of [³H]cholesterol in plasma. At the indicated times, ~25 μl of blood was collected from the tail vein of each animal. The blood samples were centrifuged to separate the RBCs from plasma, and the [³H]cholesterol in plasma was then determined by scintillation spectrometry. The results shown are means ± SEM of five animals and are representative of three independent experiments. Error bars not shown are within the symbol dimensions.

The observation that hSAA1.1/2.1₁₋₂₃ and hSAA1.1/2.1₁₋₂₀ are effective inhibitors of Mφ ACAT activity was somewhat surprising. Based on comparisons of the sequences of mSAA2.1₁₋₂₀ and mSAA1.1₁₋₂₀, we had previously concluded that the lack of influence of mSAA1.1₁₋₂₀ on ACAT activity stemmed from the IG-for-VH substitution at positions 6 and 7 (see below). Because the region in hSAA1.1/2.1₁₋₂₃ that corresponds to that in mSAA1.1₁₋₂₀ has a conservative substitution of LG for IG (see below), we expected hSAA1.1/2.1₁₋₂₃ to be inactive:

mSAA2.1₁₋₂₀, GFFSFVHEAFQGAGDMWRAY

mSAA1.1₁₋₂₀, GFFSFIGEAFQGAGDMWRAY

hSAA1.1/2.1₁₋₂₃, RSFFSFLGEAFDGDGMWRAYS

The potent ACAT-inhibiting property of hSAA1.1/2.1₁₋₂₃ suggested, among several possibilities, that the lack of ACAT-inhibiting activity exhibited by mSAA1.1₁₋₂₀ may be attributable to the absence of an N-terminal R. Such a residue may allow a salt bridge between residues R1 and D16 in hSAA1.1/2.1₁₋₂₃ and a potential salt bridge between an inserted N-terminal residue R0 and D15 of mSAA1.1₁₋₂₀. It was to this end that R-mSAA1.1₁₋₂₀ was synthesized and its ACAT-inhibiting potential assessed. Figure 7 shows that R-mSAA1.1₁₋₂₀, in contrast to mSAA1.1₁₋₂₀, is a potent inhibitor of Mφ ACAT and also prompts cholesterol efflux in cell culture (Fig. 8). mSAA2.1₁₋₂₀, which also lacks an N-terminal R, possesses ACAT-inhibitory properties, because the VH substitution allows the correct conformation for

its activity. Preliminary modeling studies indicate that R-mSAA1.1₁₋₂₀, hSAA1.1/2.1₁₋₂₃, and mSAA2.1₁₋₂₀ can all assume similar molecular conformations, an ability lacking in mSAA1.1₁₋₂₀ when the N-terminal R is absent (Z. Jia, S. P. Tam, and R. Kisilevsky, unpublished observations).

The potential therapeutic value of increasing CEH activity by transgenic means has been alluded to by others (24). Furthermore, in culture, SAA can promote lipid efflux mediated by ABCA1 (2, 25), an ATP binding cassette transporter, and unlike apoA-I, SAA can also promote lipid efflux in an ABCA1-independent manner (25). The potential therapeutic properties of mouse SAA (and homologous human) peptides is amply illustrated by the ability of these peptides to substantially prevent, and reverse, aortic lipid lesions in apoE^{-/-} mice maintained on a high-lipid diet. As shown in Fig. 1, mice receiving peptides mSAA2.1₁₋₂₀ + mSAA2.1₇₄₋₁₀₃ at the time they started on the high-lipid diet had aortas similar in appearance to those of such mice on the low-fat diet. Furthermore, because the nonnormalized mean aortic area occupied by lipid lesions after 16 days on the high-lipid diet was 1.05% and that after 44 days was 1.85%, the groups commencing treatment after 28 days on the high-lipid diet must have values between these two numbers. Moreover, the group receiving mSAA2.1₁₋₂₀ + mSAA2.1₇₄₋₁₀₃ beginning on day 28 had 0.54% of their aortas occupied by lipid lesions after 44 days, a value at least 50% below that at the start of their treatment, indicating lesion regression. Although we did not include additional controls with irrelevant peptides in the prevention and regression experiments described here, irrelevant peptides in liposomal formulation were examined for their effects on Mφ cholesterol export in vitro and in vivo in previously published work (3). Neither mSAA2.1₂₁₋₅₀ nor mSAA2.1₅₁₋₈₀ promoted cholesterol efflux from cholesterol-laden cells that had been previously established in mice (3). Furthermore, protein-free liposomes failed to prevent the development of aortic lipid lesions in apoE^{-/-} mice on atherogenic diets. In the face of these results, we chose not to include additional negative controls.

It should be emphasized that the effect of these peptides is rapid, long-lasting [a single intravenous injection appears to be effective for 96 h or more (3)], and takes place even in the face of a 3- to 5-fold increase in total plasma cholesterol. Furthermore, these peptides do not appear to significantly alter plasma lipid parameters. In this respect, synthetic small molecule ACAT inhibitors have also been shown to have this effect without reducing plasma cholesterol levels (26-28). This is not surprising, because the administration of these peptides, as liposomes, would target Mφ primarily and not the total body synthesis of cholesterol. It is precisely these cells, as foam cells, and their stored cholesterol, that play a crucial role in the pathogenesis of aortic lipid lesions. They are at the "beginning" of the reverse cholesterol pathway as it relates to cholesterol-laden Mφ. Agents (SAA peptides, or small molecule mimetics thereof) that are targeted to and prompt these cells to release their cholesterol to a natural acceptor (e.g., HDL), small though these amounts may be

relative to total circulating cholesterol, may have a profound influence on the progression of atherogenesis.

Previous work by others has demonstrated that amphipathic peptides may prevent the development of aortic lesions in hyperlipidemic mice (29–34). This raises the question of whether the M ϕ cholesterol efflux-promoting property of mSAA2.1 observed in culture and in vivo, and the antiatherogenic properties of its N and C termini observed in hyperlipidemic mice in vivo described here, are a function of amphipathic structure at its N terminus, as modeled previously (35–38). Based on published experimental data, we do not believe this is the most logical explanation for SAA2.1's antiatherogenic effects. We have demonstrated previously that the complete mSAA2.1 protein, but not the complete mSAA1.1 protein, is effective at promoting the export of cholesterol from M ϕ in culture and in vivo (2, 3). Both of these proteins contain the necessary amino acid sequence for an amphipathic N terminus, but only the 2.1 isoform has the property of influencing M ϕ cholesterol efflux. Furthermore, although both isoforms contain the postulated amphipathic domain at the N terminus, critical biological differences relevant to cholesterol mobilization exist between mSAA2.1_{1–20} and mSAA1.1_{1–20}. 1) mSAA2.1_{1–20} possesses ACAT-inhibitory properties and promotes cholesterol efflux in culture and in vivo, neither of which is observed with mSAA1.1_{1–20} (2, 3). 2) The absent ACAT-inhibitory property of mSAA1.1_{1–20} is recovered after the addition of an N-terminal R, which does not change the postulated amphipathic property already present. 3) Moreover, the C-terminal peptide, mSAA2.1_{74–103}, which has CEH-enhancing activity, does not on the basis of its amino acid sequence contain an amphipathic domain. 4) Finally, our previously published data (2, 3) demonstrated convincingly that apoA-I does not affect ACAT or CEH, even though it too has amphipathic properties. Thus, the antiatherogenic properties of the two peptides, mSAA2.1_{1–20} and mSAA2.1_{74–103}, are more consistent with their demonstrated effects on ACAT and CEH activity than on any postulated amphipathic properties. An additional region within the central portion of both SAA isoforms deserves mention, as it has apparent cholesterol binding properties (39), but peptides spanning this region fail to exert any effect in culture or in vivo on ACAT or CEH activity or on cholesterol efflux (2, 3).

Our current data and previously published work beg the question: how does SAA exert its antiatherogenic effects in vivo? And why is there a need for liposomal formulations in the prosecution of this work?

The physiological role of SAA2.1 is one directed at cholesterol efflux/recycling from M ϕ at sites of acute tissue injury. The induction of SAA is not a physiological response to atherogenesis in particular. Nevertheless, as we demonstrate in the present work, the mechanism by which SAA2.1 promotes M ϕ cholesterol efflux suggests that its active domains can be used for antiatherogenic purposes.

With regard to how SAA exerts its antiatherogenic effects in vivo, we have previously demonstrated that 1)

acute-phase mouse HDL (HDL/SAA) has a significantly higher affinity for mouse peritoneal M ϕ than normal mouse HDL (21), and 2) M ϕ from inflamed animals have a significantly increased number of binding sites for HDL/SAA (21). We and others (22, 23) have shown that HDL/SAA is readily and rapidly taken up by M ϕ , probably through a receptor-mediated mechanism. Furthermore, such HDL/SAA, when taken into cholesterol-laden M ϕ , inhibits M ϕ ACAT activity, enhances CEH activity, and promotes substantial cholesterol efflux from these cholesterol-laden cells (2, 3), and these effects are mediated through SAA2.1. Therefore, our working hypothesis is that this is the physiological role for SAA, and it is in this manner that SAA would have antiatherogenic properties in vivo. As is apparent, SAA in vivo, as part of HDL, does not require a liposomal formulation for its action.

Although there are many reasons for requiring liposomal preparations in our studies, one was to determine which HDL/SAA apolipoprotein(s) exerted the observed M ϕ effects. Other reasons focused on potential therapeutic considerations (see below).

With regard to the HDL/SAA apolipoproteins, both SAA isoforms when delipidated and purified are very insoluble in aqueous media and aggregate even at concentrations between 0 and 6 $\mu\text{g/ml}$ (data not shown). To determine which of apoA-I, SAA1.1, or SAA2.1 exerted the observed M ϕ effects on ACAT, CEH, and cholesterol export activities, these proteins were purified and, for solubility reasons, reconstituted in HDL-like liposomes (2). The results showed conclusively that SAA2.1, rather than apoA-I or SAA1.1, exercised the observed effects. SAA2.1 was then shown to contain two domains, one of which affected M ϕ ACAT activity and the other of which affected CEH activity (3).

Although, as indicated above, liposomal formulations are not required for HDL/SAA's effects on ACAT, CEH, and cholesterol export activities in vivo, they are needed for the following reasons: 1) to solubilize the purified SAA isoforms for direct study; 2) because human SAA1.1 and SAA2.1 are both amyloidogenic (40), and thus intact SAA is not useful as a potential therapeutic agent (these findings directed us to the active domains as potential therapeutic agents); 3) because naked SAA2.1 peptides are rapidly cleared from the circulation and have no effect on cholesterol export in vivo (data not shown); 4) because liposomes target the relevant peptides to M ϕ ; 5) because full-length human SAA2.1 (104 residues) in addition to being amyloidogenic is difficult to manufacture economically and with the required purity for human use; 6) and because the active domains are much shorter and therefore easier to manufacture and formulate for delivery.

Our previous and present results question the concept that SAA is a proatherogenic protein. This concept arose because correlations exist between small increases in plasma SAA concentration and poor clinical outcomes in patients with unstable angina (41, 42). Furthermore, SAA levels increase with age, and patients with the highest SAA levels are more likely to manifest cardiovascular disease (43, 44). Similar correlations have been shown in mice on

high-lipid diets (45). However, correlations, be they in humans or mice, do not establish causation. Small increases in SAA may be a consequence of lipid uptake by M ϕ when mice are on a high-lipid diet or of the local inflammatory process that is part and parcel of atherogenesis. We have argued in the past (2, 3) that it is much more likely that SAA is a marker of, rather than a cause of, vascular inflammation, a process that is now receiving much greater attention in the pathogenesis of atherosclerosis. ■■

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