Serum Amyloid A and Lipoprotein Retention in Murine Models of Atherosclerosis


Objective—Elevated serum amyloid A (SAA) levels are associated with increased cardiovascular risk in humans. Because SAA associates primarily with lipoproteins in plasma and has proteoglycan binding domains, we postulated that SAA might mediate lipoprotein retention on atherosclerotic extracellular matrix.

Methods and Results—Immunohistochemistry was performed for SAA, apolipoprotein A-I (apoA-I), apolipoprotein B (apoB), and perlecan on proximal aortic lesions from chow-fed low-density lipoprotein receptor (LDLR)^−/− and apoE^−/− mice euthanized at 10, 50, and 70 weeks. SAA was detected on atherosclerotic lesion extracellular matrix at all time points in both strains. SAA area correlated highly with lesion areas (apoE^−/−, r=0.76; LDLR^−/−, r=0.86), apoA-I areas (apoE^−/−, r=0.88; LDLR^−/−, r=0.80), apoB areas (apoE^−/−, r=0.74; LDLR^−/−, r=0.89), and perlecan areas (apoE^−/−, r=0.83; LDLR^−/−, r=0.79) (all P<0.0001). In vitro, SAA enrichment increased high-density lipoprotein (HDL) binding to heparan sulfate proteoglycans, and immunoprecipitation experiments using plasma from apoE^−/− and LDLR^−/− mice demonstrated that SAA was present on both apoA-I−containing and apoB-containing lipoproteins.

Conclusions—In chow-fed apoE^−/− and LDLR^−/− mice, SAA is deposited in murine atherosclerosis at all stages of lesion development, and SAA immunoreactive area correlates highly with lesion area, apoA-I area, apoB area, and perlecan area. These findings are consistent with a possible role for SAA-mediated lipoprotein retention in atherosclerosis.


Key Words: atherosclerosis ■ lipoproteins ■ perlecan ■ proteoglycans ■ serum amyloid A

Lipid accumulation and inflammation have been recognized as prominent features of atherosclerotic lesions. Recent studies have demonstrated an association between increased levels of plasma markers of inflammation, such as C-reactive protein and serum amyloid A (SAA), and increased cardiovascular risk. In vitro studies have suggested mechanisms through which C-reactive protein might participate in atherogenesis, such as increasing endothelial monocyte chemoattractant protein-1 expression and decreasing endothelial production of endothelial nitric oxide synthase and prostacyclin. In contrast, little attention has been paid to SAA, which is constitutively expressed in humans but not in mice. In humans and mice, plasma SAA1 and SAA2 levels can increase by several hundred-fold in response to an acute inflammatory stimulus. The majority of plasma SAA is associated with lipoproteins, primarily high-density lipoproteins (HDL), but also with very-low-density lipoproteins (VLDL). Both HDL and VLDL remnants accumulate in human atherosclerotic lesions. In the case of HDL, in vitro studies have demonstrated that proteins that may associate with HDL, including apolipoprotein E (apoE) and phospholipid transfer protein, dramatically increase the binding of HDL to extracellular matrix proteoglycans. However, apolipoprotein A-I (apoA-I), the major apolipoprotein of HDL, also is present in lesions of apoE-deficient (apoE^−/−) mice, implying that molecules other than apoE must also mediate HDL retention on atherosclerotic extracellular matrix. SAA contains proteoglycan-binding domains, raising the possibility that SAA might mediate HDL retention on vascular extracellular matrix.

The present study was undertaken to determine whether SAA is present in lesions of apoE^−/− and low-density lipoprotein receptor-deficient (LDLR^−/−) mice and, if so, whether
SAA colocalizes with apoA-I and/or apoB, as well as with perlecain, a heparan sulfate proteoglycan that is the major extracellular proteoglycan in murine atherosclerotic lesions. In addition, in vitro binding studies were performed to determine whether higher SAA content was associated with increased HDL binding to heparan sulfate proteoglycans. Also, immunoprecipitation studies were performed to determine whether SAA-containing lipoproteins from the plasma of apoE⁻/⁻ or LDLR⁻/⁻ mice also contained either apoA-I or apoB.

Methods

Animals

Sections of proximal aortic tissue were available from the majority of (though not all) mice used in a previous study evaluating the proteoglycan and apolipoprotein composition of murine atherosclerotic lesions. Briefly, LDLR⁻/⁻ and apoE⁻/⁻ mice (both strains back-crossed for at least 8 generations onto the C57BL/6 genetic background) were obtained either from the Jackson Laboratory (Bar Harbor, Me) or from colonies at the University of Washington (Seattle, Wash). The mice were fed rodent chow (Wayne Rodent BLOX 8604; Teklad Test Diets, Madison, Wis). This diet contains 4.4% fat and no added cholesterol. Mice had ad libitum access to chow and water and were housed in a temperature-controlled (25°C), modified, specific pathogen–free facility. After euthanization by intraperitoneal injection of pentobarbital (80 mg/kg), blood was collected from the retro-orbital sinus into tubes containing 1 mmol/L EDTA and used in vitro. Hearts were perfusion-fixed with 10%

H2O2

saline (n=5; apoE⁻/⁻) or 100 mmol/L CaCl2 and 2 mmol/L MgCl2 (pH 7.4; Buffer A), dialyzed extensively at 4°C against 10 mmol/L Hepes, 150 mmol/L NaCl, 5 mmol/L CaCl2, and 2 mmol/L MgCl2 (pH 7.4; Buffer A), and the protein concentrations were determined (BioRad Laboratories, Hercules, Calif) as described previously. Enzymatic assay (Liberty Scientific, #225 to 26) using cholesterol standards (Preciset 12552; Roche Molecular Biochemicals, Inc. Indianapolis, Ind) as described previously. After plasma cholesterol levels had been obtained, no remaining plasma samples were saved. Therefore, it was not possible to perform plasma SAA levels on these animals.

Quantifying Total Lesion Area and Immunostained Areas

Immunostaining was performed for apoA-I, SAA, and apoB on exactly adjacent sections from 23 apoE⁻/⁻ mice and from 29 LDLR⁻/⁻ mice. Immunohistochemistry for perlecain was performed on sections from 16 apoE⁻/⁻ mice and from 21 LDLR⁻/⁻ mice. The perlecain sections were 4 sections removed from the apoE sections, 5 sections removed from the SAA sections, and 6 sections removed from the apoA-I sections. Total lesion areas and immunostained areas for apoA-I, SAA, apoB, and perlecain were quantified from these sections using the ImagePro Plus, Version 4.5.1, software program (MediaCybernetics, Silver Spring, Md). Immunostained areas were determined using a selection of threshold for grayscale, brightness, and contrast. Results were converted to units of μm².

Heparan Sulfate Proteoglycan Isolation

Heparan sulfate proteoglycans were isolated from cultured human arterial smooth muscle cells that had been metabolically labeled with 100 μCi/mL Na[³⁵S]-sulfate. Conditioned medium was concentrated and purified by ion exchange chromatography and applied to a Sepharose CL-2B molecular sieve column. Fractions, including Kₐ 0.30 to 0.44, were taken for preparation of heparan sulfate proteoglycans (HSPGs). These pools were concentrated by ion exchange chromatography on DEAE Sephacel in 8 mol/L urea buffer, washed with 8 mol/L urea buffer lacking detergent, eluted with 4 mol/L guanidine buffer without detergent, and changed into Tris acetate buffer. Eluted material was incubated with chondroitin ABC lyase (Sigma, St Louis, Mo). The digestion product was applied to DEAE Sephacel, washed with 8 mol/L urea buffer to remove disaccharides resulting from digestion of chondroitin sulfate/dermatan sulfate, and eluted again with guanidine buffer. Eluted fractions were then dialyzed into association buffer for the gel-shift assay.

Gel-Mobility Shift Assay

Before the assay, the ³⁵S-labeled HSPG and HDL preparations were dialyzed extensively at 4°C against 10 mmol/L Hepes, 150 mmol/L NaCl, 5 mmol/L CaCl2, and 2 mmol/L MgCl2, (pH 7.4; Buffer A), and the protein concentrations were determined (BioRad Laboratories, Hercules, Calif) using bovine gamma globulin as the standard. Increasing concentrations of lipoprotein were incubated with 15–2000 pM of ³⁵S-labeled HSPG (=0.4 μg glycosaminoglycan) for 1 hour at 37°C in a total volume of 20 μL of buffer A. Electrophoretic gel-shift mobility assays then were performed as described previously.
ly. Gels were fixed in 0.1% cetyl pyridinium chloride in 70% ethanol for 90 minutes, air-dried, and the radioactive label detected by gel exposure to a PhosphorImager screen (Packard, Meriden, Conn). The amounts of HDL-bound and free HSPG in each lane were quantified for 90 minutes, air-dried, and the radioactive label detected by gel

Immunoprecipitation Experiments
SAA-containing lipoproteins were isolated from 50 μL of plasma from each of 6 mice using 5 μL of rabbit anti-mouse SAA antiserum (kind gift from Dr Godfrey Getz, University of Chicago) coupled to 100 μL of protein-G Dynal beads, according to manufacturer’s instructions (Dynal Biotech, Brown Deer, Wis); 20 μL of immunoprecipitation elutant from each mouse then was used in each subsequent immunoblotting experiment.

Statistical Analyses
All experimental data are expressed as mean±SEM. Mean values were compared using Student t test or ANOVA. Pearson correlation coefficients were calculated to examine relationships between lesion SAA immunostained areas and either total aortic sinus lesion areas or immunostained areas for apoA-I, apoB, or perlecan. P<0.05 was considered statistically significant. Statistical analyses were performed using the GraphPad Prism Program (Version 3.02, GraphPad Software, Inc, San Diego, Calif).

Results
Plasma Total Cholesterol Levels, Aortic Sinus Lesion Areas, and Presence of SAA in Plasma of LDLR−/− and ApoE−/− Mice
Plasma total cholesterol levels (mean±SEM) were slightly higher at all time points in apoE−/− as compared with LDLR−/− mice (Table). Aortic sinus lesion areas (mean±SEM) were substantially greater in apoE−/− mice, especially at the later time points (Table). At the 10-week time point in chow-fed LDLR−/− mice, only 2 of 9 animals had any detectable lesions. Lesion size was substantially higher at 70 weeks than at 50 weeks in the LDLR−/− mice. The reason for the apparent dramatic increase in lesion size between 50 and 70 weeks seen in this strain is not known; it may prove to be a reproducible finding or may be caused by chance, because the number of animals in each group is relatively low.

Early Lesions: Colocalization of SAA With ApoA-I and ApoB
In both apoE−/− and LDLR−/− strains, the earliest detectable atherosclerotic lesions consisted of subendothelial, extracellular accumulation of SAA, apo A-I, and apoB, with or without macrophages. SAA colocalized with both apoA-I (Figure 1, comparison of left and center) and apoB (Figure 1, right).

Intermediate Lesions: Colocalization of SAA With ApoA-I and ApoB
In addition, SAA and apoA-I colocalized in intermediate lesions of both apoE−/− and LDLR−/− mice (Figure 2, left and middle). As with the early lesions, apoB also was colocalized with SAA.

SAA Areas in Murine Atherosclerotic Lesions and Correlations With Apolipoprotein and Lesion Areas
In apoE−/− mice (n=23), SAA immunostained areas correlated strongly with lesion areas (r=0.76), apoA-I areas (r=0.88), and apoB areas (r=0.74) (all P<0.0001). Similarly, in LDLR−/− mice (n=29), SAA immunostained areas
correlated strongly with lesion areas (r = 0.86), apoA-I areas (r = 0.80), and apoB areas (r = 0.89) (all P < 0.0001).

Localization of SAA, ApoA-I, and ApoB With the HSPG, Perlecan, in Murine Atherosclerotic Lesions

It has been demonstrated previously that the HSPG, perlecan, colocalized with apoA-I in atherosclerotic lesions of apoE<sup>-/-</sup> mice, a surprising finding, because it also has been shown previously that only apoE-containing, but not apoE-free, HDL binds to proteoglycans in vitro. SAA immunostained areas correlated strongly with immunostained areas for the HSPG, perlecan, in both apoE<sup>-/-</sup> mice (n = 16, r = 0.83) and in LDLR<sup>-/-</sup> mice (n = 21, r = 0.79) (both P < 0.0001) (Figure 3).

Increased SAA Content Is Associated With Increased HDL Binding to HSPGs

To determine whether HDL with an increased SAA content had increased ability to bind to HSPGs, HDL was isolated from mice that had been injected with LPS to increase HDL

![Figure 2](image.png)

**Figure 2.** Intermediate (50-week) apoE<sup>-/-</sup> and LDLR<sup>-/-</sup> mouse lesions. Colocalization of SAA with apoA-I and apoB. Shown are photomicrographs of directly adjacent aortic sinus sections demonstrating colocalization of SAA with apoA-I and apoB in intermediate lesions of both strains. (original magnification = 40×, methyl green counterstain).

![Figure 3](image.png)

**Figure 3.** Late (70-week) LDLR<sup>-/-</sup> lesion. Colocalization of perlecan with SAA, apoA-I, and apoB. Shown are directly adjacent aortic sinus sections stained for SAA, apoA-I, and apoB (lower panels), as well as neighboring, but not adjacent, sections stained with the Gomori stain or for perlecan (upper panels). SAA, apoA-I, and apoB are present in the regions with intense perlecan staining (black arrows) (original magnification = 40×; Gomori stain (upper left panel) or methyl green counterstain).

![Figure 4](image.png)

**Figure 4.** Effect of SAA content on apoE<sup>-/-</sup> mouse HDL binding to heparan sulfate proteoglycans. Autoradiographs of gel from a gel-mobility shift assays in which 35S-labeled heparan sulfate proteoglycans (HSPGs) had been incubated with pooled HDL isolated from LPS-injected (A) or saline-injected (B) apoE<sup>-/-</sup> mice. Radiolabeled HSPGs bound to HDL are retained at the origin, whereas free HSPGs migrate into the gel. Greater amounts of radiolabeled HSPGs are retained at the origin for HDL from LPS-injected mice (A) as compared with saline-injected mice (B). Relative binding of LPS-HDL and saline–HDL to HSPGs is shown graphically (C). By immunoblotting, SAA content was dramatically increased in HDL from LPS-injected as compared with saline injected mice (D).

SAA content and from control saline-injected mice. HDL was isolated from apoE<sup>-/-</sup> mice to remove any potential contribution of apoE to HDL binding. In the gel-mobility shift assay, HDL isolated from LPS-injected mice had increased binding to HSPGs as compared with HDL isolated from control mice (Figure 4). By immunoblotting, the SAA content was dramatically increased in HDL from LPS-injected, as compared with saline-injected apoE<sup>-/-</sup> mice (Figure 4D).

SAA Is Present on Both ApoA-I–Containing and ApoB-Containing Lipoproteins in apoE<sup>-/-</sup> and LDLR<sup>-/-</sup> Mouse Plasma

SAA-containing lipoproteins were isolated by immunoprecipitation from plasma of chow-fed apoE<sup>-/-</sup> and LDLR<sup>-/-</sup> mice as described in Methods. Immunoblots then were performed using antisera to apoA-I or to mouse apoB-48 (kind gift from Dr Stephen Young, Gladstone Institute, San Francisco, Calif). As shown in Figure 5, SAA-containing lipoproteins from each strain include both apoA-I–containing (Figure 5A) and apoB-48–containing (Figure 5B) lipoproteins.

**Discussion**

The present study demonstrates that SAA accumulates in atherosclerotic lesions of apoE<sup>-/-</sup> and LDLR<sup>-/-</sup> mice. SAA was detected at all time points studied, consistent with the possibility that SAA might play a role at each of these stages of lesion development. In addition, lesion SAA areas correlated strongly with both lesion apoA-I areas and lesion apoB areas. There were strong correlations between lesion SAA area and total lesion area. Moreover, HDL in which SAA...
content was increased because of intraperitoneal LPS injection had increased in vitro binding to heparan sulfate proteoglycans as compared to HDL isolated from saline-injected mice. Finally, immunoprecipitation demonstrated that SAA-containing lipoproteins included both apoA-I–containing lipoproteins and apoB-containing lipoproteins. These observations are consistent with a potential role for SAA in lipoprotein retention in atherosclerotic lesions. However, these observations cannot determine whether the effect of SAA-mediated lipoprotein retention on atherogenesis might be positive, negative, or neutral.

Several recent clinical studies have demonstrated an association between elevated levels of SAA and increased risk of cardiovascular events. Epidemiologically, elevated SAA levels are associated with a number of atherosclerotic risk factors, including obesity and insulin resistance, which are features of the metabolic syndrome. However, it is unknown whether SAA is simply a marker for increased cardiac risk or actually participates in the pathogenesis of atherosclerosis. SAA are acute phase proteins synthesized by the liver, and LDL particles but also with VLDL particles. SAA has been shown to mediate binding of HDL to differentiated macrophages and endothelial cells and to impair the ability of HDL to promote cholesterol efflux from macrophages. Free SAA also has been shown to promote monocyte chemotaxis and adhesion. Thus, SAA could have multiple cellular, pro-atherogenic effects. However, SAA also may have antiatherogenic effects, because one recent study has demonstrated that the SAA isoform, SAA 2.1, can promote macrophage cholesterol efflux.

This study has focused on a potential role for SAA in mediating lipoprotein retention on extracellular matrix. Recent work has suggested that in addition to inflammation, lipoprotein retention on extracellular matrix is an early and key event in the pathogenesis of atherosclerosis. Most work in this area has focused on mechanisms involved in LDL retention on matrix. In addition to a direct interaction of LDL with matrix, molecules with the capacity to bind both lipoproteins and extracellular matrix, such as lipoprotein lipase and sphyngomyelin, can act as “bridging” molecules that further promote retention of LDL on vascular extracellular matrix. We previously have shown that apoE and phospholipid transfer protein (PLTP) also can act as bridging molecules that facilitate the retention of HDL on extracellular matrix. HDL is retained in both human and murine atherosclerotic lesions, possibly via the “bridging” roles of apoE and/or PLTP. The results of this study are consistent with a similar role in lesion HDL retention for SAA, which not only associates with HDL in plasma but also contains proteoglycan-binding domains.

SAA also may associate with plasma VLDL, particularly in inflammatory states. VLDL, which contains apoB, is retained in human atherosclerotic lesions. In the present study, there also were strong correlations between lesion SAA areas and lesion apoB areas in both strains of mice, consistent with the possibility that SAA might also enhance lesion retention of VLDL. Thus, the results of the present study suggest that SAA may be playing a role in lesion retention of both HDL and VLDL.

Retention of either VLDL or of HDL might be expected to stimulate atherosclerotic lesion formation because both would result in increased cholesterol deposition in plaques. Also, in the case of HDL, the effect might be particularly deleterious, because retained HDL would not be able to remove excess cholesterol from lesions. However, it also is possible that retained SAA might itself be antiatherogenic, for example, by promoting macrophage cholesterol efflux.

Further, it is interesting to note that as in our previous study with apoB, no SAA deposition was detected in nonlesioned areas that lacked proteoglycan accumulations. This observation is most consistent with the hypothesis that retention of lipoproteins is preceded by cellular secretion of proteoglycans, like perlecan, that have the capacity to bind lipoproteins. However, it does not exclude the possibility that, in addition to retention on proteoglycans, additional mechanisms might also contribute to lipoprotein retention in atherosclerotic plaques.

Because plasma was not available for measurement of circulating SAA levels, it was not possible to study whether plasma SAA levels correlate with atherosclerotic lesion size. However, a correlation between plasma SAA levels and lesion size would represent only indirect evidence of a role for SAA in lesion pathogenesis. In contrast, the present study demonstrates that SAA is present in atherosclerotic lesions. Further, this study has the advantage of evaluating SAA content of lesions obtained from 2 different atherosclerotic strains at three different points in time and finds that SAA is present even in the earliest detectable lesions. Therefore, the results suggest that lesion SAA is a common feature of all stages of lesion development in mouse models of atherosclerosis. However, it cannot determine whether the overall effect of SAA is to promote or to retard atherogenesis.
In summary, SAA was detected in early through late stages of lesion development in 2 mouse models of atherosclerosis. Lesion SAA area correlated strongly with lesion size and with lesion apoE-A1 area in both mouse models, and increased SAA content was associated with increased HDL binding to heparan sulfate proteoglycans. The results are consistent with a role for SAA in lipoprotein retention in atherogenesis and suggest one potential mechanism by which this plasma inflammatory “marker” may participate in atherogenesis.

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