Increase in Serum Amyloid A Evoked by Dietary Cholesterol Is Associated With Increased Atherosclerosis in Mice

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Background—Elevated serum amyloid A (SAA) levels are associated with increased cardiovascular risk. SAA levels can be increased by dietary fat and cholesterol. Moreover, SAA can cause lipoproteins to bind extracellular vascular proteoglycans, a process that is critical in atherogenesis. Therefore, we hypothesized that diet-induced increases in SAA would increase atherosclerosis independent of their effect on plasma cholesterol levels.

Methods and Results—Female LDL-receptor–null (LDLR−/−) mice were fed high–saturated fat diets (21%, wt/wt), with or without added cholesterol (0.15%, wt/wt), for 10 weeks. Compared with chow-fed controls, the high-fat diets increased plasma SAA levels. Addition of cholesterol further increased SAA levels 2-fold (P<0.05) without further increasing plasma cholesterol levels. Addition of dietary cholesterol also increased atherosclerosis (P<0.05). Four lines of evidence suggest that SAA actually might cause atherosclerosis: (1) SAA levels when mice were euthanized correlated with the extent of atherosclerosis (r=0.49; P<0.02); (2) SAA levels after 5 weeks of diet correlated with the extent of atherosclerosis at 10 weeks (r=0.66; P<0.01); (3) binding of HDL from these animals to proteoglycans in vitro was related to the HDL-SAA content (r=0.65; P<0.01); and (4) immunoreactive SAA was present in lesion areas enriched with both proteoglycans and apolipoprotein A-I, the major HDL apolipoprotein.

Conclusions—Addition of cholesterol to a high-fat diet increased plasma SAA levels and atherosclerosis independent of an adverse effect on plasma lipoproteins, consistent with the hypothesis that SAA may promote atherosclerosis directly by mediating retention of SAA-enriched HDL to vascular proteoglycans. (Circulation. 2004;110:540-545.)

Key Words: amyloid ■ inflammation ■ cholesterol ■ lipoproteins ■ diet

Inflammation plays an important role in the pathogenesis of atherosclerosis.1 Prospective studies have shown that increased levels of the inflammatory proteins C-reactive protein (CRP) and serum amyloid A (SAA) are strongly associated with an increased risk of clinical coronary events.2–4 It is not clear whether these proteins merely are markers of inflammation or whether they actually can mediate atherosclerosis. Recent studies have suggested several mechanisms by which CRP may participate directly in atherogenesis (eg, inhibition of NO synthesis,5 increased monocyte chemotactic protein-1 expression by endothelial cells,6 increased plasminogen activator inhibitor type 1 expression by endothelial cells,7 and binding to modified phospholipids in oxidized LDL and altered LDL8).

There also is increasing evidence that SAA may play a direct role in atherogenesis. For example, SAA mediates binding of HDL to differentiated macrophages and endothelial cells9 and impairs the ability of HDL to promote cholesterol efflux from macrophages.10 SAA also promotes monocyte chemotaxis and adhesion.11 Moreover, SAA is an apolipoprotein that can bind to extracellular vascular proteoglycans.12 Because of their ability to bind atherogenic lipoproteins, retention of lipoproteins by extracellular vascular proteoglycans is believed to play a critical role in atherogenesis.13–15 SAA is the major acute-phase protein in vertebrates.16 Levels can increase 100- to 1000-fold in response to acute inflammatory stimuli.12,16,17 Much lower but nonetheless elevated levels of inflammatory markers such as CRP and SAA seen in humans with obesity and the metabolic syndrome are predictive of cardiovascular disease events.16,19 Moreover, diets high in saturated fat and cholesterol, which are associated with increased risk of cardiovascular disease in humans,20 increase SAA levels in mice.21 Mice are an excellent species in which to examine the potential role of SAA in atherogenesis because CRP levels do not increase in response to inflammatory stimuli. Thus, we hypothesized that diet-induced elevations of SAA would result in increased
atherosclerosis by facilitating the retention of SAA-containing lipoproteins to extracellular vascular proteoglycans. To test this hypothesis, we developed a mouse model in which SAA levels were increased independent of adverse changes in lipids and lipoprotein levels. Findings from this study are consistent with a causal role for SAA in atherogenesis.

**Methods**

**Animals and Diets**
Six- to 8-week-old female LDL-receptor–null (LDLR−/−) mice bred onto a C57BL/6 background (Jackson Laboratories, Bar Harbor, Me) were fed ad libitum chow diets (Wayne Rodent BLOX, Teklad; 4% fat, wt/wt; 0.04% cholesterol, wt/wt) or diets rich in saturated fat (21%, wt/wt), with or without added cholesterol (0.15%, wt/wt; Harlan-Teklad), for 10 weeks. The baseline cholesterol content of the saturated fat–rich diet was 0.05% (wt/wt). There were 10 chow-fed LDLR−/− mice and 15 LDLR−/− mice in each of the high-fat-diet groups. Animals were housed in cages equipped with microisolator filter tops, maintained on a 12-hour light/dark cycle in a temperature-controlled room, and weighed weekly. These studies were conducted under Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines and were approved by the University of Washington Animal Care Committee.

After the animals had fasted for 4 hours, blood was obtained via the retro-orbital sinus at baseline, after 5 weeks of feeding the diets, and immediately before the mice were euthanized. Blood was collected into tubes containing 1 mmol/L sodium EDTA (final concentration) and kept on ice, and plasma was obtained by centrifugation. After 10 weeks of diet treatment, mice were killed by carbon dioxide asphyxiation and immediately before the mice were euthanized. Blood was collected into tubes containing 1 mmol/L sodium EDTA (final concentration) and kept on ice, and plasma was obtained by centrifugation. After 10 weeks of diet treatment, mice were killed by carbon dioxide asphyxiation and immediately before the mice were euthanized.

**Quantification of SAA and Serum Amyloid P Levels**
SAA levels were measured by ELISA in plasma and in fast-performance liquid chromatography (FPLC) fractions of individual mice, as well as in HDL from pools of 3 mice.22 Purified recombinant mouse SAA1 was used as the standard, and the primary antibody was a polyclonal antibody generated in rabbits against recombinant mouse acute-phase SAA1 (kind gifts from Dr Godfrey Getz, University of Chicago). Diluted samples and standards were allowed to bind to 96-well ELISA plates (Costar) for 2 hours at 37°C. Plates were washed 5 times with PBS, and nonspecific sites were blocked with 3% (wt/vol) BSA in PBS for 1 hour at 37°C. The primary antibody (diluted 1:2000 in blocking buffer) was added and incubated for 1.5 to 2 hours at 37°C, followed by 5 washes with PBS. Plates were then incubated in the presence of anti-rabbit horseradish peroxidase–conjugated IgG (Boehringer-Mannheim, diluted 1:4000 in blocking buffer) for 1 hour at room temperature and washed 5 times with PBS, and the color reaction was developed with o-phenylene diamine as the substrate (Sigma). Sulfuric acid was added to wells (1.6N, final concentration) to terminate the reaction, and the plates were read at wavelengths 490 to 405 nm. Another inflammatory marker, serum amyloid P (SAP), which has structural similarities to CRP, also was measured by ELISA.23

**High-Density Lipoproteins**
HDL (d=1.063 to 1.21 g/mL) was isolated by density-gradient ultracentrifugation from pooled plasma from 3 mice in each diet group for evaluation of its ability to bind the purified 35SO4-labeled extracellular proteoglycan biglycan with an electrophoretic gel mobility shift assay.25 Biglycan was isolated from cultured arterial smooth muscle cells, as described previously,26 and was chosen because it appears to play an important role in trapping lipoproteins in atherosclerotic lesions in mice27 and humans.28

**Plasma Lipids and Lipoprotein Profiles**
Plasma from individual mice was analyzed for total cholesterol and triglyceride concentrations with colorimetric assay kits (for cholesterol, Diagnostic Chemicals Limited; for triglycerides, Roche Diagnostics). To obtain a more detailed characterization of lipoproteins and cholesterol, plasma lipoprotein profiles were analyzed in 5 to 6 individual mice from each diet group by FPLC. Briefly, 100 μL of plasma was chromatographed on a Superose 6 HR10/30 column (Amersham Pharmacia Biotech) and eluted with PBS, 0.02% sodium azide, pH 7.4. Sixty 0.5-ml fractions were collected, and fractions 11 to 41 were analyzed for cholesterol and SAA content. Values for the amount of cholesterol within each of the lipoprotein classes were determined by quantifying the area under the curve from each lipoprotein profile with fractions 15 to 20 for VLDL and IDL, fractions 21 to 27 for LDL, and fractions 28 to 35 for HDL.

**Quantification of Atherosclerosis**
Atherosclerosis was evaluated both by analysis of serial sections at the aortic sinus and by en face analysis of the aortic arch, as described previously.29 Briefly, hearts were fixed with 10% formalin and embedded in paraffin, and a series of at least 30 10-μm sections were taken beginning at the junction of the left ventricle and the aorta. A total of 5 sections from each of 5 to 8 LDLR−/− mice from each diet group were stained with Movat’s pentachrome, as described previously,30 to identify proteoglycans, which stain blue. Average lesion area was quantified with ImagePro-Plus software (Media Cybernetics). For en face analysis at the aortic arch (n=5 to 6 in each strain and diet group), the remainder of the aorta was stripped of adventitia and opened longitudinally from the iliac arteries to a point equidistant between the origin of the brachiocephalic artery and the aortic root, followed by removal of the branching vessels. The formalin-fixed aorta was pinned out flat on a black wax surface, and the sudanophilic lesion area could be visualized easily without staining. Area measurements were quantified with ImagePro-Plus software.

**Immunohistochemistry**
Immunohistochemical detection of SAA in atherosclerotic lesions in the LDLR−/− mice was performed with the same rabbit polyclonal antibody against recombinant mouse SAA1 that was used in the ELISA (diluted 1:200). Goat polyclonal antiserum raised against human apolipoprotein (apo) A-1 (dilution 1:1000; a kind gift from Dr John Oram, University of Washington) is reactive against mouse apoA-1.23

**Statistical Analyses**
Values shown are mean±SEM. Significance of differences between means was assessed by Student’s t test and ANOVA. Significant relationships were analyzed by multiple linear regression analyses. Probability values <0.05 were considered statistically significant.

**Results**

**Plasma Lipids and Lipoproteins**
After 10 weeks, LDLR−/− mice fed the high-fat diets had ≈5-fold higher cholesterol levels than chow-fed controls, but the addition of 0.15% cholesterol to the high-fat diet did not further increase cholesterol levels (Figure 1). Both high-fat diets increased HDL cholesterol and triglyceride levels compared with the chow diet, although triglyceride levels were lower on the high-fat diet with added cholesterol (Table 1). The addition of cholesterol to the high-fat diet resulted in a shift of cholesterol from the LDL fraction to the VLDL/IDL fraction. HDL cholesterol levels did not change with the addition of cholesterol to the diet.
Plasma SAA and SAP Levels

Before the diets were begun, plasma SAA levels in the mice were low (4.3 ± 0.1 μg/mL). Significant increases in SAA occurred after 5 weeks of feeding the high-fat diet or the high-fat diet with cholesterol compared with the Chow diet (P < 0.005; Table 2). At this 5-week time point, very minor fatty streaks would just be beginning to develop in LDLR−/− mice.27 SAA levels at 10 weeks also differed significantly in the LDLR−/− mice fed the Chow and high-fat diets (P < 0.05) but had not increased beyond levels observed at 5 weeks. The addition of 0.15% cholesterol to the high-fat diet was associated with 2-fold higher SAA levels at 10 weeks relative to the high-fat diet without added cholesterol (Table 2). This suggests that dietary cholesterol, rather than fat, is the major determinant of SAA levels in these hypercholesterolemic animals. Pooled data from the 2 high-fat diet-fed LDLR−/− groups showed no correlation between plasma levels of SAA and total cholesterol (r = 0.24, P = NS). Data “clustering” for cholesterol precluded comparison of the relationship between SAA and cholesterol levels for the individual diet groups. SAP levels were not affected by diet (33.6 ± 2.5, 39.1 ± 3.5, and 36.3 ± 2.8 μg/mL for the Chow-fed, high-fat, and high-fat-plus-cholesterol groups, respectively).

Lipoprotein Distribution of SAA

Analysis of SAA distribution among FPLC fractions in the LDLR−/− mice showed that the majority of SAA was present on HDL in Chow-fed mice, although some SAA was present in fractions other than HDL (data not shown). However, with either of the high-fat diets, ~40% of total plasma SAA was present in the VLDL/IDL fraction, and to a lesser extent LDL (Figure 2). No difference in the distribution of SAA was apparent between the LDLR−/− mice fed the high-fat diets with or without added cholesterol. More than 95% recovery of plasma SAA was from the FPLC fractions (data not shown).

Atherosclerotic Lesion Analyses

Compared with chow-fed LDLR−/− mice, mean aortic sinus lesion areas were increased >100-fold with the high-fat diet and >200-fold with the high-fat–plus–cholesterol diet (Table 3). For en face aortic arch lesion areas, highly significant increases of >100-fold and >250-fold were seen, respectively, for the high-fat and the high-fat–plus–cholesterol diet groups (P < 0.02 and P < 0.001 versus Chow, respectively; Table 3). Although there was a trend toward larger lesions in the high-fat group with added cholesterol, the differences failed to reach statistical significance because of large variances.

Lesion Size Correlates With Plasma SAA but Not With Plasma Cholesterol Levels

In LDLR−/− mice, plasma SAA levels correlated significantly with the extent of atherosclerosis at the aortic arch (r = 0.49; P < 0.02; Figure 3). Moreover, SAA levels obtained after 5 weeks on diet correlated significantly with 10-week lesion areas at the aortic sinus (r = 0.66, P < 0.01), which suggests both that SAA levels predict atherosclerosis extent in LDLR−/− mice and that plasma SAA might play a direct role

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**TABLE 1. Effect of Diets on Plasma Lipid and Lipoprotein Levels in LDLR−/− Mice**

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Total Cholesterol, mg/dL</th>
<th>VLDL/IDL Cholesterol, mg/dL</th>
<th>LDL Cholesterol, mg/dL</th>
<th>HDL Cholesterol, mg/dL</th>
<th>TG, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>177 ± 4</td>
<td>22 ± 10</td>
<td>67 ± 5</td>
<td>78 ± 5</td>
<td>46 ± 3</td>
</tr>
<tr>
<td>High fat</td>
<td>830 ± 26*</td>
<td>347 ± 36*</td>
<td>333 ± 11*</td>
<td>107 ± 19*</td>
<td>584 ± 67*</td>
</tr>
<tr>
<td>High fat + cholesterol</td>
<td>886 ± 23*</td>
<td>458 ± 31†</td>
<td>233 ± 13†</td>
<td>111 ± 23†</td>
<td>243 ± 19†</td>
</tr>
</tbody>
</table>

TG indicates triglycerides.
Values shown are mean ± SEM.
*P < 0.05 vs Chow diet.
†P < 0.05 vs high-fat diet.

**TABLE 2. Plasma SAA Levels Increase in Response to High-Fat Diets in LDLR−/− Mice**

<table>
<thead>
<tr>
<th>Time</th>
<th>Chow (n=5), μg/mL</th>
<th>HF (n=10), μg/mL</th>
<th>HF and Cholesterol (n=10), μg/mL</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 weeks</td>
<td>8.4 ± 1.2</td>
<td>34.8 ± 5.2</td>
<td>55.9 ± 8.2</td>
<td>0.005</td>
</tr>
<tr>
<td>10 weeks</td>
<td>5.7 ± 1.1</td>
<td>28.5 ± 7.7</td>
<td>61.5 ± 16.0</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values shown are mean ± SEM. The number of mice is shown in parentheses.
*One-way ANOVA.
Discussion

We found that the addition of dietary cholesterol to a high-fat diet led to an increase in circulating SAA levels. SAA levels in turn correlated strongly with the extent of atherosclerosis in LDLR−/− mice, independent of an adverse effect on plasma lipids and lipoproteins. CRP levels are not affected by diet or inflammation in mice,16,31 thus eliminating a role for this inflammatory marker. The lack of an increase in SAP also eliminates this inflammatory protein as a potential contributor to atherosclerosis.

LDLR−/− mice fed either the high-fat diet or the high-fat diet with added cholesterol had similar levels of plasma cholesterol. Furthermore, the addition of cholesterol to the high-fat diet led to relatively minor changes in cholesterol distribution among lipoprotein fractions, which could not account for the difference in atherosclerosis seen between the groups. In fact, plasma triglyceride levels were significantly lower in animals fed the high-fat diet plus cholesterol, yet this group had the most atherosclerosis. Moreover, plasma cholesterol levels were not correlated with lesion areas, consistent with our previous findings.29 However, the addition of cholesterol to the high-fat diet resulted in greater SAA levels in plasma, greater immunoreactive SAA area in lesions, and greater atherosclerotic lesion areas. Moreover, plasma SAA concentrations significantly correlated with atherosclerotic lesion area, consistent with SAA playing a causal role in
atherosclerosis. Plasma SAA values obtained from LDLR<sup>−/−</sup> mice after 5 weeks of feeding the diets correlated with lesion areas quantified 5 weeks later, which suggests that SAA levels predict the extent of subsequent lesion development and that SAA may participate actively in the pathogenesis of atherosclerosis. The levels of SAA observed in mice fed the high-fat diets were much lower than those observed during an acute inflammatory response and are similar in magnitude to levels observed in clinical studies in which they have been shown to increase the risk of cardiovascular disease. Thus, these modest elevations of SAA appear to reflect a chronic inflammatory response that is likely to be pathophysiologically relevant.

The present study also demonstrates a potential mechanism by which SAA might participate directly in atherogenesis, ie, by mediating lipoprotein binding to vascular proteoglycans. SAA contains proteoglycan-binding regions, and lipoprotein binding to vascular proteoglycans has been found to play a critical role in the pathogenesis of atherosclerosis. Consistent with this, we showed that HDL SAA levels correlated with the extent of HDL binding to biglycan binding in vitro. Moreover, the in vivo observations demonstrating the presence of large amounts of SAA in atherosclerotic lesions in LDLR<sup>−/−</sup> mice, which colocalized with apoA-I and proteoglycans, suggest that SAA-mediated retention of lipoproteins on vascular proteoglycans facilitates atherogenesis. Nonetheless, it is conceivable that the diets increased both SAA and atherosclerosis via independent mechanisms. Therefore, proof that SAA directly mediates atherosclerosis must await the creation of a transgenic mouse strain that overexpresses SAA without adversely affecting plasma lipid and lipoprotein levels.

In the present study, essentially all of the SAA was associated with lipoproteins. In nonhyperlipidemic mice, SAA is present on HDL, which is the major lipoprotein class present. However, in LDLR<sup>−/−</sup> mice fed high-fat diets, a significant proportion of plasma SAA was present on VLDL/IDL fractions, similar to other apolipoproteins such as apoE and the C apolipoproteins. Although the addition of cholesterol to the high-fat diet was associated with a lowering of plasma triglycerides for reasons that are not clear, the cholesterol content of the VLDL/IDL peak separated by FPLC was increased, which suggests compositional changes in this class of lipoproteins. Unlike HDL, VLDL/IDL was not isolated and tested for its ability to bind proteoglycans in vitro in the present study, but it is conceivable that SAA-enriched remnant lipoproteins would have increased affinity for proteoglycans. By enhancing the retention of VLDL/IDL by vascular proteoglycans, the presence of SAA on those lipoproteins might also have contributed to the increased atherosclerosis seen in animals fed the high-fat diet with added cholesterol, despite their having lower plasma triglyceride levels.

These findings also suggest that HDL particles that contain SAA might be atherogenic, in contrast to the bulk of HDL particles, which are believed to have antiatherogenic properties. Plasma levels of SAA are much lower than those of apoA-I, which suggests that only a small proportion of HDL particles contain SAA. However, SAA-containing HDL could be retained by vascular proteoglycans in a manner analogous to the retention of apoB- and apoE-containing lipoproteins, after which they could undergo oxidative and other potentially atherogenic modifications. Consistent with this notion is the presence of apoA-I, the major apolipoprotein of HDL, in atherosclerotic lesions from both humans and atherosclerosis-prone mouse strains.

An atherogenic diet previously has been shown to increase SAA levels in C57BL/6 mice. The diet used in that study contained very high levels of cholesterol and cholic acid. The diet in the present study is more reflective of a Western diet, with a moderate content of cholesterol and no cholic acid. Our results are of interest in that they show that a moderate intake of dietary cholesterol against a background of a high-fat diet can result in an inflammatory response, as reflected by high SAA levels.
Together, these findings support the idea that diet, inflammation, and SAA are causally linked in the development of atherosclerosis. An increase in SAA levels mediated by a diet high in fat and cholesterol could provide a link between diet, inflammation, and atherosclerosis.

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References
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