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Serum amyloid A3 is pro-atherogenic

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Abstract

Background and aims—Serum amyloid A (SAA) predicts cardiovascular events. Overexpression of SAA increases atherosclerosis development; however, deficiency of two of the murine acute phase isoforms, SAA1.1 and SAA2.1, has no effect on atherosclerosis. SAA3 is a pseudogene in humans, but is an expressed acute phase isoform in mice. The goal of this study was to determine if SAA3 affects atherosclerosis in mice.

Methods—*ApoE*$^{-/-}$ mice were used as the model for all studies. SAA3 was overexpressed by an adeno-associated virus or suppressed using an anti-sense oligonucleotide approach.

Results—Over-expression of SAA3 led to a 4-fold increase in atherosclerosis lesion area compared to control mice (p=0.01). Suppression of SAA3 decreased atherosclerosis in mice genetically deficient in SAA1.1 and SAA2.1 (p<0.0001).

Conclusions—SAA3 augments atherosclerosis in mice. Our results resolve a previous paradox in the literature and support extensive epidemiological data that SAA is pro-atherogenic.

Keywords

inflammation; atherosclerosis; murine models

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Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Author contributions

The study was conceived and designed by LRT, NRW and FCdB; the experiments were performed by JCT, PGW, AJ, PS and MdB.

Introduction

Serum amyloid A (SAA) is a family of acute phase proteins that predict cardiovascular events[1, 2]. In normal healthy individuals, SAA concentrations are low, typically < 5 mg/L. However, in chronic inflammatory conditions such as obesity, diabetes, or rheumatologic diseases (all of which are associated with increased cardiovascular disease) SAA is chronically elevated. SAA exerts a number of pro-atherogenic activities including induction of IL-1β and TNF-α[3], promotion of monocyte and neutrophil chemotaxis[4], upregulation of vascular proteoglycan synthesis and increased lipoprotein retention[5], and promotion of thrombosis[6]. We previously demonstrated that overexpression of human SAA1 (plasma concentrations ~30 mg/L; similar to levels in obese/ diabetic humans[7]) significantly increased atherosclerotic lesion area in *apoE*$^{-/-}$ mice[8]. Similarly, Dong et al. used a lentivirus to overexpress murine SAA1.1 in *apoE*$^{-/-}$ mice (achieving plasma levels of ~35 mg/L) and reported increased atherosclerosis[9]. Paradoxically, we later showed that targeted deletion of the two major acute phase isoforms, SAA1.1 and SAA2.1, did not alter atherosclerosis development in *apoE*$^{-/-}$ mice[10].

SAA is a family of homologous isoforms that likely arose from gene duplication. *SAA* genes are highly conserved across species, each comprising 4 exons that encode the 104–112 amino acid secreted SAA. The genes for the two major acute phase human SAAs, *SAA1* and *SAA2*, correspond to mouse *Saa1.1* and *Saa2.1* based on their relative map positions and transcriptional orientations. Whereas mice express a third acute phase SAA, SAA3, this isoform is not expressed in humans due to the presence of a premature stop codon. Both human *SAA4* and mouse *Saa4* are constitutively expressed at relatively low levels and are not acute phase reactants. As SAA3 is a pseudogene in humans, this isoform has largely been overlooked in atherosclerosis studies. In this study, we demonstrate for the first time that SAA3 contributes to atherosclerotic lipid deposition in mice.

Materials and methods

Animals

All studies were approved by the Institutional Animal Care and Use Committee and conducted in accordance with Public Health Service policy on humane care and use of laboratory animals. *ApoE*$^{-/-}$ mice on the C57BL/6 background (Jackson Laboratories, stock #002052) were the murine model for all studies. Mice deficient in SAA1.1 and SAA2.1 crossed to *apoE*$^{-/-}$ were generated as previously described[10]. Mice deficient in SAA1.1, SAA2.1 and SAA3 (*SAA1.1/2.1/3-TKO*) mice were generously provided by Drs. June-Yong Lee and Dan Littman, New York University. The *SAA1.1/2.1/3-TKO* mice were generated by inserting a premature stop codon into exon 2 of *saa3* in the *SAA1.1/2.1-DKO* mouse using CRISPR-Cas9 technology. AAV vectors (serotype 8) were produced by the Viral Vector Core at the University of Pennsylvania. The AAV-SAA3 vector contains an insert expressing mouse Serum Amyloid A3 (GenBank accession NM_011315). Empty AAV vector (null AAV) was used as control. For the AAV study 8–10 week old male *apoE*$^{-/-}$ mice were injected via lateral tail vein with AAV-SAA3 or a control AAV at 1 ×10e11 particles in a total volume of 100 μl. Blood samples (non-fasted) were collected from cheek vein at 0 and 6 weeks. Mice were fed normal rodent chow throughout the study. After 12

weeks mice were killed and blood and tissues were collected for analysis. For the ASO study 8–10 week old male *apoE*$^{-/-}$ × *SAA1.1/2.1-DKO* mice were injected with 25 mg/kg ASOs a total of 4 times over 2 weeks. The ASO to SAA3 and control ASO were provided by Ionis Pharmaceuticals. Western diet was initiated 2 days after the last injection (Envigo, Indianapolis, IN; diet #TD.88137). Mice were bled via cheek vein at 0 and 6 weeks (non-fasted), then killed after 12 weeks of diet with blood and tissues collected for analysis. All mice were housed in specific pathogen free housing with 12h light/dark cycles and had ad libitum access to food and water.

Immunohistochemistry

Immunohistochemistry for SAA was performed on sections of the aortic roots from mice fed an atherogenic diet as previously described[10] or sections of livers from mice 24 h after injection with LPS (1 μg/gm body weight) using normal rabbit serum (011-000-001, Jackson Immuno Research, West Grove, PA, 1:200 dilution) or a monospecific rabbit polyclonal antibody against mouse SAA (1:200 dilution, de Beer laboratory). Smooth muscle alpha actin (SMA; goat anti-human, Novus Biologicals, Littleton, CO, Cat#NB300-978) was used as an assay control antibody at a concentration of 10 mg/ml. The secondary biotinylated antibodies against SAA and SMA (711-065-152 and 705-065-147, respectively, Jackson Immuno Research,1:500 dilution) and avidin-peroxidase were subsequently incubated with the sections (Vectastain Elite ABC kit cat no PK-6101, Vector Laboratories Inc., Burlingame, CA). Immunoreactivity was visualized using an AEC substrate kit (cat no SK-4200, Vector Laboratories Inc., Burlingame, CA).

Assays

SAA1.1/2.1 and SAA3 were measured by ELISA kits (SAA1/2 ELISA: cat #TP 802 M, Tridelta Development Ltd, Kildare, Ireland; SAA3 ELISA cat# EZMSAA3-12K, Millipore, Billerica, MA). Total plasma cholesterol and triglycerides were measured by enzymatic assay kits (Wako, Richmond, VA). TNF-α and IL-6 were measured by ELISA kits (R&D Systems, Minneapolis, MN).

Atherosclerosis

For quantification of atherosclerosis, adventitial tissue was removed from aortas then aortas were cut open longitudinally and pinned *en face* to expose intimal surfaces. Atherosclerotic lesions were analyzed in the region from the ascending aorta to 3mm distal to the subclavian artery. Atherosclerosis quantifications were measured in duplicate by observers blinded to the group assignments using ImagePro Plus software (Media Cybernetics, Bethesda, MD) as previously described[11].

RNA isolation and quantitative RT-PCR

Total RNA was isolated from mouse livers according to the manufacturer's instructions (RNeasy® Mini Kit, cat no 74106, Qiagen). RNA samples were incubated with DNase I (cat no 79254, Qiagen) for 15 min at RT prior to reverse transcription. Liver RNA (0.5 μg) was reverse transcribed into cDNA using the Reverse Transcription System (cat no 4368814, Applied Biosystems CA). After 4-fold dilution, 5 μl was used as a template for real-time RT-

PCR. Amplification was done for 40 cycles using Power SYBR Green PCR master Mix Kit (cat no 4367659, Applied Biosystems). Quantification of mRNA was performed using the ΔΔCT method and normalized to *GAPDH*. Primer sequences are, *GAPDH* (NM_008084), 5'-CTCATGACCACAGTCCATGCCA-3' (F) and 5'-GGATGACCTTGCCCACAGCCTT-3' (R); SAA3 (NM_011315), 5'-TTTCTCTTCCTGTTGTTCCCAGTC-3' (F) and 5'-TCACAAGTATTTATTCAGCACATTGGGA-3' (R).

Statistical analyses

Statistical differences were evaluated by two-tailed Mann-Whitney test. $p < 0.05$ was considered statistically significant.

Results

We previously reported that *apoE*$^{-/-}$ mice deficient in SAA1.1 and SAA2.1 (*apoE*$^{-/-}$ × *SAA1.1/2.1-DKO*) were not protected from atherosclerosis[10]. We now show immunopositive SAA staining in lesions of *apoE*$^{-/-}$ × *SAA1.1/2.1-DKO* mice, suggesting that SAA3 is present in lesions of mice lacking SAA1.1 and SAA2.1 (Fig. 1A). The specificity of our polyclonal anti-SAA antibody was confirmed by staining livers from LPS-injected *C57BL/6, SAA1.1/2.1-DKO*, and *SAA1.1/2.1/3-TKO* mice (Fig. 1B). Male *apoE*$^{-/-}$ mice were injected with an AAV expressing SAA3 or a null virus and fed normal rodent chow for 12 weeks. Blood was collected at 0, 6 and 12 weeks after AAV injections. Plasma SAA1.1/2.1 did not differ between groups (~13mg/L). SAA3 was significantly higher in the AAV-SAA3 group compared to control AAV group (Fig. 1C; p=0.0003). TNF-α did not differ between groups but IL-6 was significantly higher in the AAV-SAA3 mice (7.3±2.9 pg/ml) compared to control AAV mice (0.7± 1.2 pg/ml; p=0.03). Total plasma cholesterol (~18 mmol/L) and triglycerides (~1.5 mmol/L) did not differ between groups. Mice injected with AAV-SAA3 had 3–4-fold increased atherosclerotic lesion area compared to control AAV or saline treated mice (Fig. 1D; p=0.01).

To determine if suppression of SAA3 alters atherosclerosis development *apoE*$^{-/-}$ × *SAA1.1/2.1-DKO* mice were injected with an antisense oligonucleotide (ASO) to SAA3 or a control ASO then fed an atherogenic Western diet for 12 weeks. RT-PCR on livers confirmed suppression of SAA3 mRNA in the SAA3-ASO group (p=0.016; Fig. 1E). TNF-α did not differ between groups but IL-6 was significantly lower in SAA3-ASO mice (undetectable) compared to control-ASO mice (6.9±4.1 pg/ml; p=0.02). Total plasma cholesterol (~30 mmol/L) and triglycerides (~4.5 mmol/L) did not differ between groups. Mice injected with the SAA3-ASO had a significant ~50% reduction in atherosclerosis (Fig. 1F; p<0.0001).

Discussion

Robust epidemiological as well as in vitro data support the concept that acute phase SAAs are pro-atherogenic. We and others previously demonstrated that over-expression of SAA1 led to increased atherosclerosis in apoE$^{-/-}$ mice.[8, 9] However, deficiency of SAA1.1/2.1 in apoE$^{-/-}$ mice had no effect on atherosclerosis, thus disputing a role for SAAs in lesion

development[10]. SAA3 is a third acute phase SAA in mice that is induced in the liver, adipocytes and macrophages during acute inflammation[12, 13]. Mouse SAA3 shares considerable homology with the major adipocyte and liver derived human SAA1[13]. However, as SAA3 is not expressed in humans, we and others have tended to overlook the role of SAA3 in mice. The major findings of this brief report are: 1) atherosclerotic lesions in *apoE*$^{-/-}$ × *SAA1.1/2.1-DKO* mice are immunopositive for SAA, indicating that SAA3 is found in murine atherosclerosis; 2) AAV-mediated SAA3 expression increases atherosclerosis in *apoE*$^{-/-}$ mice; and 3) ASO-mediated SAA3 suppression reduces atherosclerosis in *apoE*$^{-/-}$ mice lacking SAA1.1/SAA2.1. Of note, changes in circulating IL-6 levels accompanied changes in SAA3 expression: whereas increased SAA3 expression was accompanied by increased IL-6, suppression of SAA3 resulted in suppression of IL-6. This data is compatible with abundant in vitro data that SAA induces inflammatory cytokine expression by signaling through pattern recognition receptors[14] and the report by Dong et al that retroviral vector mediated SAA1 expression increased circulating IL-6[9]. Taken together, these new data resolve the previous apparent discrepancies in the literature regarding SAA and atherosclerosis, and provide conclusive evidence that beyond serving as a biomarker of cardiovascular disease, SAA isoforms play a causal and perhaps redundant role in atherosclerosis development.

Although SAA3 is not expressed in humans, it is expressed in mice and several studies have concluded that it is an acute phase reactant in mice[12, 13]. Mice are commonly used as in vivo models for studies investigating mechanisms underlying human atherosclerosis. The apparent conflict in the literature between increased atherosclerosis in SAA over-expression models[8, 9] compared to the lack of effect on atherosclerosis in mice deficient in SAA1.1/2.1[10] raised major concerns about the validity of mice as a model for human atherosclerosis, and cast doubt on the role of SAA in atherogenesis. However, we now demonstrate that SAA3 plays a role in atherogenesis, at least in *apoE*$^{-/-}$ mice. Thus, our findings have re-validated mice as a model for studying atherosclerosis mechanisms. Furthermore, the collective results of studies examining an atherogenic role for SAA have found that only modest increases in SAA concentrations above baseline lead to increased atherosclerosis[8, 9] and that suppression/deficiency of SAA are atheroprotective (current data). These findings have particular relevance to individuals with chronic inflammation, where plasma SAA is chronically albeit modestly elevated. We propose that the chronic elevation of SAA in humans with conditions such as obesity, metabolic syndrome, and diabetes may be an underlying cause of the increased cardiovascular disease observed in these populations. However, our results also point out that murine studies investigating SAA must consider all 3 acute phase isoforms of SAA. Further work is needed to investigate potential differences in tissue distributions and/or functions of the different isoforms. We are currently crossing our recently developed *SAA1.1/2.1/3-TKO* mice with *apoE*$^{-/-}$ mice for comprehensive mechanistic studies, with the goal of understanding the pathobiological effects of these highly conserved acute phase reactants.

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Highlights

- Increased expression of SAA3 increases atherosclerosis development
- Suppression of SAA3 in mice deficient in SAA1.1 and SAA2.1 attenuates atherosclerosis development
- SAA3 has pro-atherogenic activity in mice

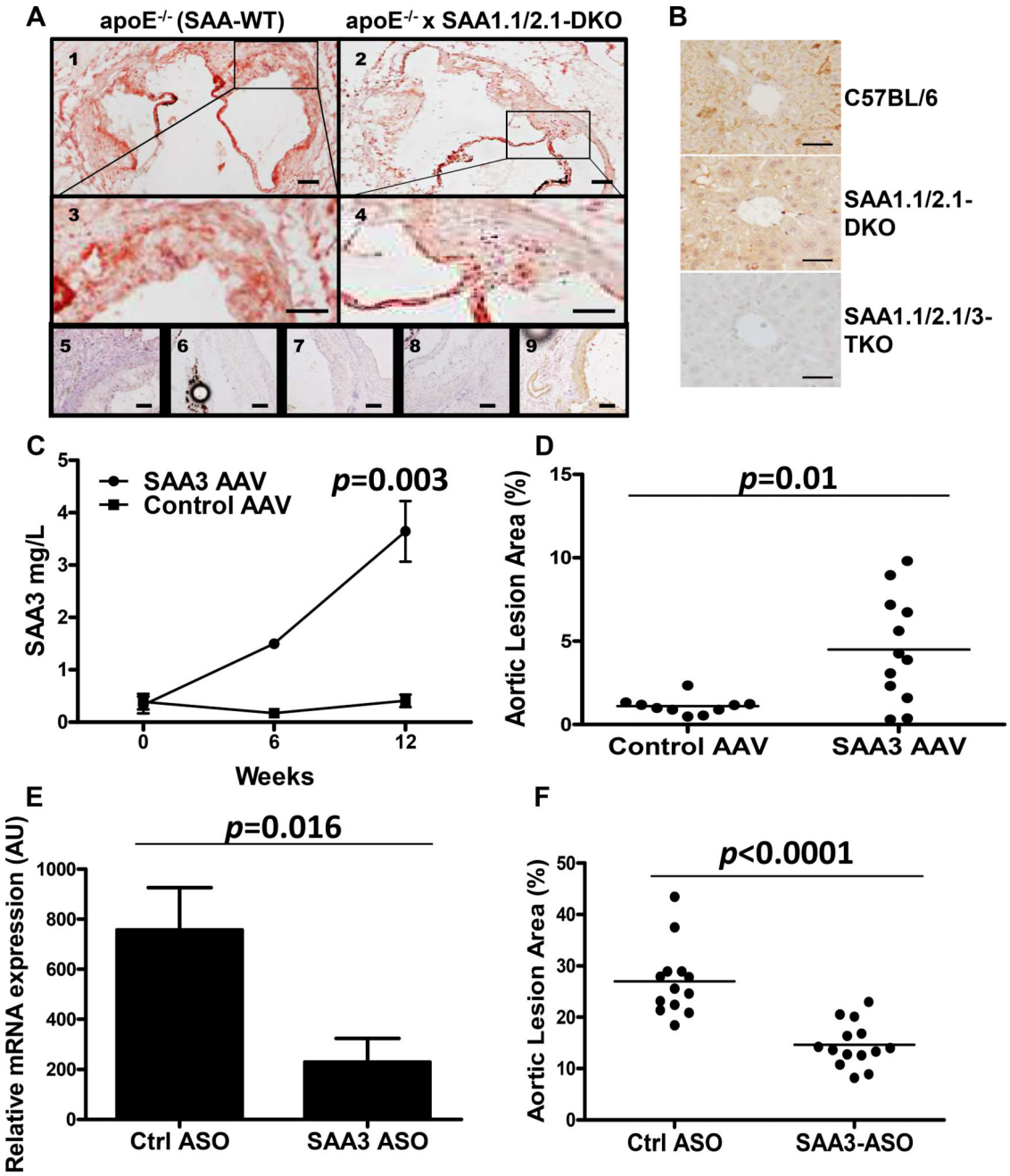

Fig. 1. SAA3 is pro-atherogenic

(A) *ApoE*$^{-/-}$ [SAA wildtype (WT)] or *apoE*$^{-/-}$ × *SAA1.1/2.1-DKO* mice were fed a western diet for 12 weeks as described in[10]. Aortic root sections from *apoE*$^{-/-}$ (panels 1,3) or *apoE*$^{-/-}$ × *SAA1.1/2.1-DKO* (panels 2,4) mice fed a western diet for 12 weeks were stained for SAA. Shown are aortic root images representative of n=3/group magnified 10× with insets (panels 3,4) magnified 25×. Scale bars are 100 μm. Panels 5–9 are control *apoE*$^{-/-}$ sections: IgG only, no primary, no secondary, no Ab, and SMA positive control respectively. (B) Liver sections from LPS-injected *C57BL/6, SAA1.1/2.1-DKO* and *SAA1.1/2.1/3-TKO* mice were stained for SAA. Images shown are magnified 40×; scale bars are 250 μm. (C) *ApoE*$^{-/-}$

mice were injected with a control AAV or AAV expressing SAA3 and fed normal chow for 12 weeks. Plasma SAA3 was measured at weeks 0, 6, 12. Data shown is mean±SEM. (D) Atherosclerosis was quantified on the *en face* aorta, each symbol represents the atherosclerotic area for an individual mouse; horizontal lines represent the group mean. (E) *apoE*$^{-/-}$ × *SAA1.1/2.1-DKO* mice were injected with a control ASO or an ASO to SAA3 and fed western diet for 12 weeks. *SAA3* mRNA abundance in livers (arbitrary units, AU). Data shown is mean±SEM. (F) Atherosclerosis was quantified on the *en face* aorta, each symbol represents the atherosclerotic area for an individual mouse; horizontal lines represent the group mean (E).