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Serum amyloid A stimulates macrophage foam cell formation via lectin-like oxidized low-density lipoprotein receptor 1 upregulation

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ABSTRACT

Elevated levels of serum amyloid A (SAA) is a risk factor for cardiovascular diseases, however, the role of SAA in the pathophysiology of atherosclerosis remains unclear. Here we show that SAA induced macrophage foam cell formation. SAA-stimulated foam cell formation was mediated by c-jun N-terminal kinase (JNK) signaling. Moreover, both SAA and SAA-conjugated high density lipoprotein stimulated the expression of the important scavenger receptor lectin-like oxidized low-density lipoprotein receptor 1 (LOX1) via nuclear factor-κB (NF-κB). A LOX1 antagonist carrageenan significantly blocked SAA-induced foam cell formation, indicating that SAA promotes foam cell formation via LOX1 expression. Our findings therefore suggest that SAA stimulates foam cell formation via LOX1 induction, and thus likely contributes to atherogenesis.

1. Introduction

Atherosclerosis is a chronic inflammatory disease affecting arterial blood vessels. Several types of inflammatory cells are involved in the atherogenic process, including monocyte/macrophages and smooth muscle cells [1]. Circulating monocytes migrate into the intima region in response to chemokine (C–C motif) ligand 2 (CCL2) [2] where macrophage colony-stimulating factor stimulates their differentiation into macrophages [3]. Macrophages engulf modified low-density lipoprotein (LDL), resulting in foam cell formation [4]. Foam cells produce diverse growth factors and proinflammatory mediators including tumor necrosis factor (TNF)-α [4,5]. These inflammatory mediators induce proliferation of smooth muscle cells, which induce plaque formation [6]. Given the importance of foam cell formation in the pathogenesis of atherosclerosis [5,7], understanding the underlying molecular mechanisms of foamy macrophage generation may lead to novel targets for therapeutic intervention. Previous studies have reported that several Toll-like receptors (TLRs) including TLR2, TLR4, and TLR9 are involved in foam cell formation: TLR ligands pam3csk, lipopolysaccharide (LPS), and CpG ligands can independently stimulate foam cell formation from macrophages [8–10]. Targeting the TLRs may, however, have undesirable effects on host immune defense. Thus the identification of additional stimuli and their receptors involved in foam cell formation may provide superior targets for therapeutic intervention.

Serum amyloid A (SAA), a major acute-phase protein, is released into blood circulation in response to infection or injury [11]. SAA is released after liver cells are stimulated by several pro-inflammatory cytokines, such as interleukin (IL)-1β or tumor necrosis factor (TNF)-α [12,13]. Circulating SAA levels are elevated approximately 1000-fold during acute-phase reactions compared with baseline conditions [12]. SAA has cytokine-like properties and plays a number of immunomodulatory roles. SAA induces proinflammatory cytokine and chemokine production in several cell types such as rheumatoid synoviocytes, intestinal epithelial cells, monocytes, and neutrophils [13–16]. SAA has also been suggested to play a role in the metabolism of high density lipoproteins (HDL), and as an impeder of the protective function of HDL on the development of atherosclerosis [17]. We previously demonstrated that SAA stimulates many atherogenic inflammatory markers, including CCL2, matrix metalloproteinase (MMP)-9 in monocytes and endothelial cells [18–21]. Importantly, circulating SAA levels are significantly elevated in patients with chronic vascular disease reaching around

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11–15 μg/ml or in animals fed with high fat diet reaching around 28.5–34.8 μg/ml [22,23]. However it is unclear whether elevated SAA levels reflect underlying cardiovascular disease or can contribute directly to the pathophysiology of atherogenesis. Here we show that SAA directly stimulates foamy macrophage formation and thus implicate SAA as causal agents in atherogenesis.

2. Materials and methods

2.1. Materials

Recombinant human SAA (catalog number 300-13, produced in *Escherichia coli,* endotoxin level <0.1 ng/μg) was purchased from Peprotech (Rocky Hill, NJ, USA). Naïve LDL, LPS (from *E. coli* 055:B5, catalog number L2880) and carrageenan (type III kappa) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All antibodies against the phospho-MAPKs were purchased from Cell Signaling Technology (Beverly, MA, USA), and the anti-lectin-like oxLDL receptor 1 (LOX1) antibody (catalog number AF1564) was purchased from R&D Systems (Minneapolis, MN, USA).

2.2. Cell culture and generation of bone marrow-derived macrophages

Raw264.7 cells were maintained in DMEM with 10% heat-inactivated fetal calf serum under standard incubator conditions (humidified atmosphere, 95% air, 5% CO_2, and 37 °C). Bone marrow cells were isolated by flushing the femurs and tibias of wild-type ICR mice 5–8 weeks of age with ice-cold PBS. Bone marrow progenitor cells were cultured in 10% FBS containing α-MEM with 30 ng/ml M-CSF under standard incubator conditions for 3 days. The non-adherent cells were removed, and 10% FBS containing α-MEM with 30 ng/ml M-CSF was added, and the cells maintained for 2–3 days.

2.3. Foam cell formation and oil red O staining

Raw264.7 cells and mouse bone marrow-derived macrophages (1×10^4) were seeded on 96-well plates and cultured overnight. Cells were stimulated with LDL (50 μg/ml) plus vehicle, SAA, or LPS for 24 h. After washing with PBS, the cells were fixed with 4% formaldehyde for 10 min at room temperature. After washing with distilled water 3 times, the fixed cells were stained with oil Red-O solution for 20 min. The stained cells were detected by light microscopy and total cells and foam cells were counted.

2.4. Western blot analysis

Raw264.7 cells were stimulated with SAA for various times. After stimulation, the cells were lysed in lysis buffer (20 mM HEPES [pH7.2], 10% glycerol, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Soluble proteins were separated on 10% SDS–polyacrylamide gels and blotted onto a nitrocellulose membrane. Subsequently, the membranes were incubated with specific antibodies against target protein, and antigen–antibody complexes were visualized by enhanced chemiluminescence.

2.5. Reverse transcription polymerase chain reaction (RT-PCR) analysis

Raw264.7 cells or mouse bone marrow-derived macrophages (1×10^6) were stimulated with SAA for the indicated times. Total RNA was isolated by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and 1 μg of total RNA was used as a template for cDNA using the Bioneer Reverse Transcriptase System. The primers used for the RT-PCR analyses have been reported previously. The sequences of the primers were as follows: LOX1: sense, 5′-AGGTCCTTGTCCACAAGACTGG-3′; antisense, 5′-ACGCCCCTGGTCTTAAAGAATTG-3′. actin: sense, 5′-TTCTTTGCAGCTCCTTCGTTGCCG-3′; antisense, 5′-TGGATGGCTACGTACATGGCTGGG-3′. GAPDH: sense, 5′-GACATCAAGAAGGTGGTGAA-3′; antisense, 5′-TGTCATACCAGGAAATGAGC-3′. cDNA was subjected to 35 PCR cycles at 94 °C (denaturation, 30 s), 55–65 °C (annealing, 30 s), and 72 °C (extension, 30 s). PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining.

2.6. Transfection of oilgodeoxynucleotides (ODN)

The sequence of phosphorothionate double-stranded decoy ODN against the NF-κB binding site was as follow: NF-κB decoy ODN (5′-CCTTGAAGGGATTTCCCTCC-3′/3′-GGAACTTCCCTAAAGGGAGG-5′), scrambled NF-κB ODN (5′-TTGCCGTACCTGACTTAGCC-3′/3′-AACGGCATGGACTGAATCGG-5′). Single-stranded ODN was annealed for 2 h while the temperature was decreased from 80 to 25 °C. Cells (3×10^5) were seeded on a 24-well plate and cultured overnight. The cells were transfected with 0.2 μg of NF-κB decoy ODN and scrambled NF-κB ODN using Lipofectamine 2000 reagent.

2.7. Luciferase assay

NF-κB reporter constructs were purchased from Clontech (Palo Alto, CA, USA). Raw264.7 cells were transfected with 2 μg of plasmid construct by the Lipofectamine method (Invitrogen). After transfection, cells were stimulated with 1 μM SAA for 24 h and lysed with lysis buffer; 5 μl of cell lysate was mixed with 25 μl of luciferase activity assay reagent, and the luminescence produced for 5 s was measured using Luminoskan (Labsystems).

2.8. Synthesis of reconstituted HDL and HDL-conjugated SAA

Human apolipoprotein A-I (apoA-I) was expressed and purified according to a previous report [24]. Discoidal reconstituted HDL was prepared with the purified apoA-I (at least 95% purity) as described previously [25]. HDL-SAA was prepared with apoA-I and SAA via sodium cholate dialysis with a POPC: cholesterol: apoA-I: SAA: sodium cholate molar ratio of 95:5:1:1:150. All HDL showed a similar low range of residual endotoxin level between 3.1–3.3 EU/mL based on endotoxin quantification using a commercially available test kit (BioWhittaker, Walkersville, MD, USA).

2.9. Data analysis

Results are expressed as mean ± SE. The Student's *t*-test was used to compare individual treatments with their respective control values. Statistical significance was set at $p < 0.05$.

3. Results

3.1. SAA promotes foam cell formation

To investigate the effect of SAA on foam cell formation, we stimulated the mouse macrophage model cell line Raw264.7 with SAA and LDL for 24 h. SAA treatment significantly induced foam cell formation in a concentration-dependent manner (Fig. 1A and B). The increase in foam cell formation was apparent with exposure to 0.1–2 μM SAA (Fig. 1B). As a positive control, treatment with LPS and LDL also induced foam cell formation (Fig. 1A and C), which is consistent with a previous report [9]. Because the recombinant SAA was produced in *E. coli*, we examined the possible contribution of contaminating LPS to SAA-induced foam cell formation using polymyxin B, a potent LPS inhibitor. While polymyxin B