Serum Amyloid A Activates Nuclear Factor-κB in Rheumatoid Synovial Fibroblasts Through Binding to Receptor of Advanced Glycation End-products

HIROSHI OKAMOTO, YUKIKO KATAGIRI, AKIKO KIIRE, SHIGEKI MOMOHARA, and NAOYUKI KAMATANI

ABSTRACT. ***Objective.*** Rheumatoid arthritis (RA) is a chronic, symmetric polyarticular joint disease and serum amyloid A (SAA) is an acute-phase protein that is upregulated during the course of RA. We investigated the role of SAA in the pathogenesis of RA.

Methods. Fibroblast-like synovial cells (FLS) were established from RA joints. SAA-stimulated expression of cytokines from FLS was evaluated by ELISA. Nuclear factor-κB (NF-κB) activation by SAA was evaluated by luciferase assay. NF-κB activation and IκBα degradation were evaluated by Western blotting and nuclear localization of p65 subunit of NF-κB in FLS. Expression of receptor for advanced glycation end-products (RAGE) in synovial tissue was evaluated by immunohistochemical study. Effects of preincubation of soluble RAGE on NF-κB activation by SAA was evaluated by Western blotting of IκBα.

Results. SAA stimulated the transcriptional activation by NF-κB in a dose-dependent manner and induced expression of the proinflammatory cytokines interleukin 6 (IL-6) and IL-8. Higher expression of RAGE in synovial tissue from patients with RA was noted. SAA induced IκBα degradation, with the peak effect around 30 minutes. Preincubation of SAA with soluble recombinant RAGE protein prevented SAA-induced IκBα degradation. SAA stimulation promoted nuclear translocation of NF-κB, whereas preincubation of SAA with RAGE inhibited nuclear translocation.

Conclusion. Our data suggested that the SAA-RAGE-stimulated NF-κB signaling pathway has an important role in the pathogenesis of RA. (First Release Mar 1 2008; J Rheumatol 2008;35:752–6)

Key Indexing Terms:

RECEPTOR FOR ADVANCED GLYCATION END-PRODUCT

SERUM AMYLOID A RHEUMATOID ARTHRITIS

AA amyloidosis is mainly encountered as a complication of chronic inflammatory diseases, and rheumatoid arthritis (RA) is the most frequent cause[1,2]. Serum amyloid A (SAA) is an acute-phase protein produced by hepatocytes in response to proinflammatory cytokines, and its expression is upregulated during the course of the inflammatory process[3]. Amyloid is formed from an amyloidogenic precursor protein that is present in excess amounts as a result of its increased production[4]. Therefore, it is likely that elevated concentrations of circulating SAA are critical in the pathogenesis of AA amyloidosis[5]. It is thought that adequate control of inflammation during the course of rheumatoid arthritis (RA) may prevent the development of AA amyloidosis[6]. Although a wealth of information concerning the diagnosis and pathogenesis of AA amyloidosis has accumulated, the biological role(s) of SAA in the pathogenesis of RA is still not fully understood.

In an intriguing study, Mullan, *et al* reported that acute-phase SAA (A-SAA) was as effective in increasing the time- and dose-dependent expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) as interleukin 1ß (IL-1ß) and tumor necrosis factor-α (TNF-α)[7]. Importantly, they demonstrated that A-SAA-induced expression of VCAM-1, ICAM-1, and matrix metalloproteinase-1 (MMP-1) was partially mediated by NF-κB signaling. They concluded that A-SAA induces expression of proteins involved in adhesion, angiogenesis, and matrix degradation, all processes that are mediated by NF-κB[7].

The accumulation of advanced glycation end-products (AGE), S100A12, and high-mobility group box chromosomal protein 1 (HMGB1) has been associated with joint inflammation in rheumatoid arthritis (RA). The receptor for these proteins, termed receptor for AGE (RAGE), has been reported to be highly expressed in synovial tissue macrophages from patients with RA[8]. RAGE has also been reported to be a receptor for the amyloidogenic form of SAA[9].

From these findings, we hypothesized that A-SAA could

From the Institute of Rheumatology, Tokyo Women's Medical University, Tokyo, Japan.

H. Okamoto, MD, PhD; Y. Katagiri; A. Kiire, MD; S. Momohara, MD, PhD; N. Kamatani, MD, PhD, Institute of Rheumatology, Tokyo Women's Medical University.

Address reprint requests to Dr. H. Okamoto, Institute of Rheumatology, Tokyo Women's Medical University, 10-22 Kawada-cho, Shinjuku, Tokyo 162-0054, Japan. E-mail: hokamoto@ior.twmu.ac.jp

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bind to RAGE on the surface of synovial cells, thereby resulting in NF-κB signaling and the active promotion of RA-mediated joint inflammation. In this study, we investigated the role of A-SAA in pathogenesis of RA; we studied the role of SAA in NF-κB activation and cytokine expression *in vitro*.

MATERIALS AND METHODS

Isolation and culture of fibroblast-like synovial cells (FLS). The tissue was obtained under aseptic conditions, and was finely minced. FLS were also isolated by the following digestion process: 1 mg/ml collagenase for 3 h at 37°C in Dulbecco's modified Eagle's medium (DMEM) and antibiotics. Digested tissue was briefly centrifuged, and the pellet was washed 3 times. Isolated cells were seeded at high density in tissue culture flasks and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Tissue Culture Biologicals, Tulare, CA, USA) and antibiotics at 37°C in a humidified atmosphere of 5% CO_2/95% air. The culture medium was changed every 3–5 days, and nonadherent lymphoid cells were removed. At confluence, FLS were detached and passaged once, and then seeded at high density and allowed to grow in DMEM supplemented as above. Only third- to seventh-passage cells were used for the following experiments.

Measurement of cytokine levels. Cytokine concentrations in FLS culture supernatants were determined using cytokine-specific ELISA kits for IL-6 and IL-8 (QuantiGlo Human IL-6/IL-8 Immunoassay, R&D Systems, Minneapolis, MN, USA). Concentrations of IL-6 and IL-8 were measured in culture supernatants after incubation with 10 ng/ml of interleukin 1ß (IL-1ß) or various concentrations of recombinant human Apo-SAA protein (PeproTech EC, London, UK) for 12 h. Absence of lipopolysaccharide contamination in recombinant human Apo-SAA protein was guaranteed by the manufacturer. The cytotoxic effects of these compounds were evaluated by measuring the number of viable cells using the 3-(4,5-demethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Experiments were performed 3 times with each of 3 independent cultures.

Luciferase assay. 293T cells are an established cell line of human fibroblasts from kidney, and we confirmed the expression of RAGE in these cells. 293T cells were transfected with 1 µg of reporter plasmid (p4xκB-Luc)[10] with lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendation, as described[11]. After transfection, cells were incubated with 10 ng/ml of IL-1ß for 48 h. Cells were lysed in a buffer containing 250 mM Tris/HCl (pH 7.5) and 0.1% Triton X-100, sonicated, and the lysate was clarified by centrifugation at 12,000 g for 15 min. As an internal control, pRL-TK, expressing *Renilla* luciferase under the control of TK promoter from herpes simplex virus, was cotransfected, and the luciferase activity was normalized by the *Renilla* luciferase activity.

Immunofluorescent staining. For immunofluorescent studies, FLS were fixed in 4.5% paraformaldehyde in phosphate buffered saline (PBS), permeabilized with 0.5% Triton X-100, and then incubated with a mouse monoclonal antibody against the p65 subunit of NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 45 min at 37°C. After washing with PBS, the cells were incubated with FITC-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology) for 20 min at 37°C.

Western blot analysis. Western blot analysis was performed by standard methods. All incubations with antibodies were for 1 h at room temperature. To study the effect of A-SAA on degradation of IκBα, cells were treated with 10 µg/ml SAA with or without preincubation with various concentrations of recombinant RAGE protein (1–4 µg/ml RAGE with 10 µg/ml SAA in 1 ml medium). Recombinant human Apo-SAA protein (PeproTech EC) and recombinant RAGE protein (Cosmobio Co., Tokyo, Japan) were used in this study. An anti-IκBα antibody (SC-371; Santa Cruz Biotechnology) was used for detection of IκBα. A rabbit polyclonal anti-GAPDH antibody (Santa Cruz Biotechnology) and anti-rabbit IgG-HRP (Santa Cruz Biotechnology) were used for detection of human GAPDH as a control.

RESULTS

Clinical studies have shown that SAA is one of the most sensitive indicators of inflammation, and SAA has been reported to be expressed in synovial cells[12-15]. To study the biological implication of SAA expression in RA chondrocytes, we further analyzed the *in vitro* effects of SAA: we studied the effects of SAA on cytokine production from FLS. As shown in Figure 1A, SAA induced expression of the proinflammatory cytokines IL-6 and IL-8 in a dose-dependent manner. To determine the effects of SAA, we transfected the p4xκB-Luc plasmid into 293T cells and treated them with IL-1ß and various concentrations of SAA. SAA stimulated

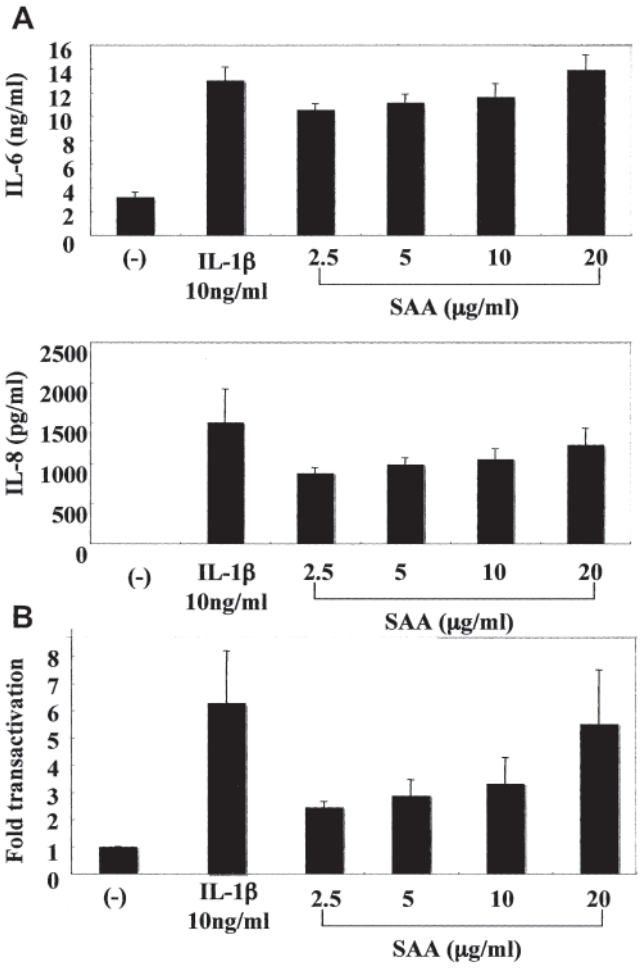

Figure 1. Cytokine expression and NF-κB activation by SAA. A. Concentrations of IL-6 and IL-8 were measured in culture supernatants after incubation with 10 ng/ml of IL-1ß or various concentrations of recombinant SAA for 12 h. B. 293T cells were transfected with 1 µg of reporter plasmid (p4×κB-Luc) by lipofectamine, then cells were incubated either in the presence of 10 ng/ml IL-1ß or various concentrations of recombinant SAA for 48 h. Cells were harvested and extracts were prepared for the luciferase assay. Data are presented as fold-increase in luciferase activity (mean ± SD) relative to the control of 3 independent transfections.

the transcriptional activation by NF-κB in a dose-dependent manner (Figure 1B). Next, we studied the effects of SAA in NF-κB activation in FLS. As RAGE has also been reported to be a receptor for the amyloidogenic form of SAA, we assumed that RAGE is expressed on synovium in patients with RA and has an important role in the pathogenesis of RA[9]. To determine expression of RAGE on synovium, we performed immunohistochemical studies. As shown in Figure 2, expression of RAGE in synovial tissue from RA patients was noted when compared to osteoarthritis synovial tissue. To study the effects of SAA on NF-κB activation, we performed Western blotting of IκBα on FLS treated with 10 μg/ml of recombinant SAA. A time-course study of IκBα degradation revealed that the peak effect of SAA stimulation occurred around 30 minutes (Figure 3A). As expected, SAA induced degradation of IκBα as well as IL-1ß (10 ng/ml). To gauge whether the effect of SAA on NF-κB activation is mediated through the binding of SAA to RAGE on synovial cells, we preincubated SAA with various concentrations of soluble recombinant RAGE protein before adding it to the FLS. We observed a dose-dependent inhibition of SAA-induced IκBα degradation (Figure 3B). As a control experiment, we also treated FLS with RAGE alone. As expected, RAGE alone did not affect the expression of IκBα (Figure 3C). Unexpectedly, higher amounts of RAGE alone inhibited the expression of IκBα by an unknown mechanism. To confirm NF-κB activation in FLS, we conducted immunofluorescence studies with a monoclonal antibody against the p65 subunit of NF-κB. As shown in Figure 3D, SAA stimulation promoted nuclear translocation of NF-κB, whereas preincubation of SAA with RAGE inhibited nuclear translocation.

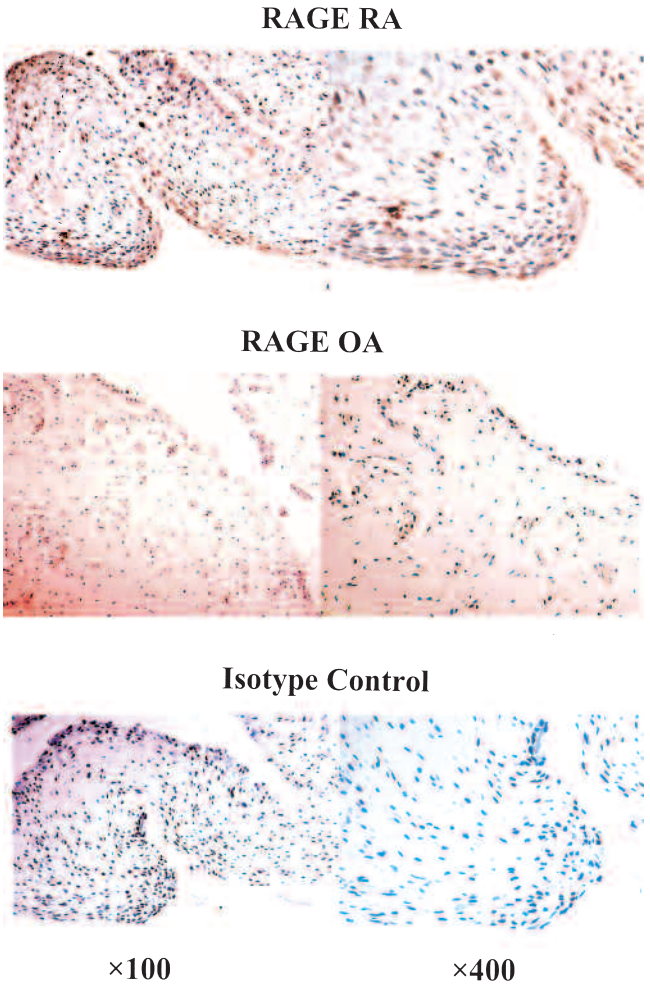

Figure 2. Immunohistochemical study of expression of RAGE in synovium. Immunohistochemical staining with an anti-human RAGE mouse monoclonal antibody. Expression of RAGE protein is shown in brown.

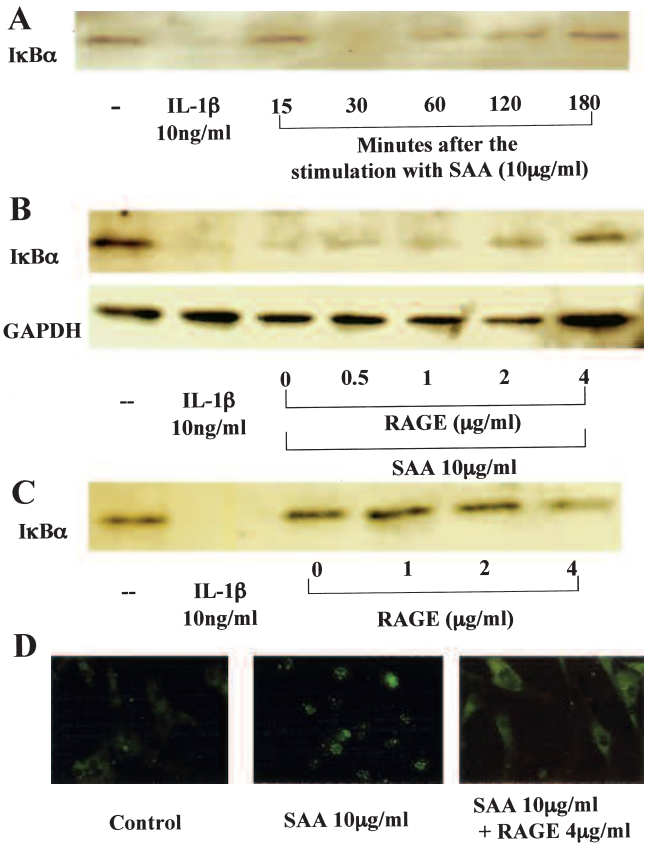

Figure 3. Role of SAA-RAGE axis in NF-κB activation. A. FLS were treated with SAA (10 μg/ml) for 15 to 180 min. Western blot analysis was performed with a specific monoclonal antibody against IκBα. B. FLS were treated for 30 min with SAA (10 μg/ml) preincubated with various concentrations of soluble recombinant RAGE protein (0–4 μg/ml). As a positive control, FLS were treated with IL-1ß (10 ng/ml) for 30 min. Western blot analysis was performed with a monoclonal antibody against IκBα. As a control, anti-GAPDH antibody was used with the same cell lysates. C. FLS were treated with various concentrations of soluble recombinant RAGE protein alone (0–4 μg/ml). Western blot analysis was performed with a monoclonal antibody against IκBα. D. Subcellular location of NF-κB in FLS was studied by immunohistochemical staining using a monoclonal antibody against the p65 subunit of NF-κB. After stimulation with SAA, NF-κB localized to the nucleus. Preincubation with soluble recombinant RAGE protein (4 μg/ml) prevented SAA-induced NF-κB nuclear translocation.

DISCUSSION

We report for the first time that intraarticular SAA could activate NF-κB signaling in FLS through binding to cell surface RAGE. These data suggested that SAA of RA joints is actively involved in the pathogenesis of RA through the SAA-RAGE-NF-κB signaling pathway. Mullan, *et al* reported that acute-phase SAA induced expression of proteins involved in adhesion, angiogenesis, and matrix degradation mediated by NF-κB[7]. Our results indicate that the activation of NF-κB by SAA previously observed by Mullan, *et al* may have occurred through the SAA-RAGE-NF-κB signaling pathway. Therefore intraarticular SAA is suggested to have an active role in the pathogenesis of RA.

NF-κB is an inducible transcription factor controlled by signal activation cascades. NF-κB controls a number of genes involved in immunoinflammatory responses, cell-cycle progression, inhibition of apoptosis, and cell adhesion, thus promoting chronic inflammatory responses. NF-κB has been reported to be constitutively activated in some rheumatic conditions such as RA. Interestingly, a number of anti-RA compounds have been shown to exhibit anti-NF-κB activities, and therapeutic strategies manipulating NF-κB and its signaling cascade have been suggested as feasible novel molecular targets in RA[16-18].

AGE, S100/calgranulins, HMGB1, amyloid-ß peptides, and the family of ß-sheet fibrils have been shown to contribute to a number of chronic diseases such as diabetes, amyloidoses, inflammatory conditions, and tumors by promoting cellular dysfunction through binding to cell-surface RAGE, which is a multiligand receptor of the immunoglobulin superfamily. The involvement of RAGE in pathophysiologic processes has been demonstrated in murine models of chronic disease using a receptor decoy such as soluble RAGE (sRAGE) and RAGE-neutralizing antibodies, and in RAGE–/– mice[19]. Several lines of evidence suggest that not only is RAGE a multiligand receptor, but its ligands are also likely to recognize several receptors in mediating their biologic effects[20]. Binding of AGE to its receptor RAGE induces the release of proinflammatory cytokines such as TNF-α or IL-6[21]. Most of these cytokines are transcriptionally activated by NF-κB. Several reports have shown that RAGE mediates NF-κB activation. The ligation of RAGE with either of the pathophysiologically relevant ligands, AGE or amyloid-peptide, is suggested to result in generation of cellular oxidative stress and activation of the transcription factor NF-κB[22,23]. In the vascular system, RAGE-mediated activation of NF-κB has been shown to induce the expression of genes such as VCAM-1, which might contribute to the development of diabetic vascular disease[24]. In neurons, RAGE-mediated activation of NF-κB in response to amyloid-peptide has been shown to induce expression of macrophage-colony-stimulating factor (M-CSF), strengthening the inflammatory response in brains of patients with Alzheimer disease[25].

In addition to the fact that binding of AGE to RAGE activates NF-κB signaling, TNF-α activates RAGE expression through NF-κB activation on the promoter region of the RAGE gene. AGE, TNF-α, and 17ß-estradiol (E_2) upregulated RAGE mRNA and protein levels in human microvascular endothelial cells and ECV304 cells[26].

These combined data suggest there is a self-perpetuating cycle among AGE, RAGE, NF-κB signaling, and cytokines. Thus, inhibition of this cycle might have therapeutic potential. One therapeutic strategy is the use of a truncated form of the receptor sRAGE. sRAGE has been suggested to function as a decoy abrogating cellular activation, but its endogenous activity is not fully understood. Pullerits, *et al* studied the properties of sRAGE *in vivo* and *in vitro* to determine the role of sRAGE in HMGB1-induced arthritis. They found that sRAGE had proinflammatory properties, and this effect was triggered by interaction with the leukocyte ß2 integrin Mac-1 and was mediated via NF-κB. Indeed, they found that systemic treatment with sRAGE significantly downregulated HMGB1-triggered arthritis, but the observed effect was due to a deviation of the inflammatory response from the joint to the peritoneal cavity rather than a genuine antiinflammatory effect. They concluded that sRAGE interacts with Mac-1 and acts as an important proinflammatory and chemotactic molecule[27]. Therefore, sRAGE may not be a feasible strategy for treatment of RA.

Recently, Lee, *et al* demonstrated that SAA promotes the proliferation of FLS, and SAA protects FLS against the apoptotic death induced by serum starvation, anti-Fas IgM, and sodium nitroprusside through binding of SAA to the formyl peptide receptor-like 1 (FPRL1) receptor[28]. These results indicate that SAA-RAGE or SAA-FPRL1 might be a potential target for the control of RA. Further studies are needed to clarify how SAA works in the pathophysiology of RA, and to develop potential therapeutic strategies for targeting the molecules involved in this pathway.

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