Mini Review

Analysis of cytokine-driven serum amyloid A expression based on the clinical results of IL-6 blocking therapy: a new *cis*-acting mechanism of STAT3

Keisuke Hagihara[1], Teppei Nishikawa[2], Atsumi Matsumura[3], Jian Song[2], Toshio Tanaka[1] and Kazuyuki Yoshizaki[1,2,*]

[1]Department of Respiratory Medicine, Allergy, Rheumatic disease, Osaka University Graduate School of Medicine, Osaka, Japan
[2]Health Care Center, Osaka University, Osaka, Japan
[3]Osaka University Graduate School of Medicine, Medical Science, Osaka, Japan

   Anti-cytokine therapies have been used for rheumatoid arthritis (RA) and their efficacy has been previously reported. Serum amyloid A (SAA) is an acute phase protein and known as a precursor of amyloid fibril in AA amyloidosis. IL-6 blocking therapy normalized the serum levels of SAA, in RA patients, while anti-TNFα or IL-1 therapy did not. From the clinical point of view, the exact identification of the induction mechanism of SAA is important. We found that IL-6 plays a critical role in the synergistic induction of the SAA gene by combining with proinflammatory cytokines. Moreover, we discovered that STAT3 plays an essential role in cytokine-driven SAA gene expression even though no typical STAT3 response element (RE) is located in its promoters. STAT3 and NF-κB p65 first form a complex following IL-1 and IL-6 stimulation, after which STAT3 interacts with non-consensus sequences at a 3'-site of NF-κB RE on the SAA gene promoter. STAT3 then recruits p300, which coordinates the interaction of NF-κB p65, STAT3 and C/EBP β in the synergistic induction of the SAA gene. Our findings have a direct bearing on a better understanding of inflammatory status and are expected to contribute to the development of a therapeutic strategy for AA amyloidosis.

＊Correspondence should be addressed to:
Kazuyuki Yoshizaki, Department of Respiratory Medicine, Allergy, Rheumatic disease, Osaka University Graduate School of Medicine, 2-2 Yamada-Oka, Suita, Osaka 565-0871, Japan. Phone: +81-6-6879-3831, Fax: +81-6-6879-3839, e-mail: kyoshizaki@hpc.cmc.osaka-u.ac.jp

Introduction

   Anti-cytokine therapies have been used for rheumatoid arthritis (RA) and their efficacy has been previously established in some clinical trials[1,2]. Serum amyloid A (SAA), an acute phase protein, and mainly produced in the liver, is well known as a precursor of amyloid A proteins in AA (secondary) amyloidosis,

which is a serious complication of RA[3]. The human SAA family consists of SAA1, SAA2, and SAA4. The first two, also known as acute phase SAAs, dramatically increase by as much as 1000 times during inflammation[4]. It has further been reported that the SAA2 gene is induced by nuclear factor κB (NF-κB) and CAAT enhancer-binding protein β (C/EBPβ) in response to stimulation by interleukin-1 (IL-1) together with interleukin-6 (IL-6) (IL-1 +6)[5].

However, this induction model does not fully explain certain clinical results, which showed that anti-IL-6 therapy, but not anti-tumor necrosis factor α (TNFα) or IL-1 therapy, normalized the serum level of SAA[1,2]. For this reason, we investigated the exact induction mechanism of SAA by proinflammatory cytokines, and especially focused on the SAA1 gene, which is reportedly as a main amyloidogenic factor in AA amyloidosis[4]. We previously discovered a new *cis*-acting mechanism of signal transducers and activators of transcription 3 (STAT3)[6,7]. The study reported here constitutes a new clinical research approach, and has a direct bearing on a better understanding of the pathogenesis of inflammatory status.

IL-6 plays a critical role in the synergistic induction of the SAA gene by proinflammatory cytokines

We first established an SAA isoform real-time quantitative RT-PCR assay system to examine the clinical results of anti-IL-6 receptor antibody therapy[6]. We then used this assay system to examine the effects of various combinations of proinflammatory cytokines, and found that IL-6 and IL-1 or IL-6 and TNFα, but not IL-1 and TNFα, synergistically induced SAA1 gene expression. These results showed that IL-6 is necessary for the synergistic induction of SAA gene by combining with IL-1, and TNFα (Fig.1A). Next, we confirmed this finding with the aid of specific inhibitors, namely, the anti-IL-6 receptor monoclonal anti-body (anti-IL-6R Mab), the IL-1 receptor antagonist (IL-1 ra), and the anti-TNFα monoclonal anti-body (anti-TNFα Mab). Fig.1B shows that anti-IL-6R Mab, but not IL-1 ra or anti-TNFα Mab, eliminated the synergistic induction of SAA in response to triple stimulation of IL-6, IL-1, and TNFα. Almost the same results were obtained when three typical hepatic cell lines, HepG2, Hep3B, and PLC/PRF/5, were used. We show here representative data for HepG2 cells. These results indicate that IL-6 plays a critical role in the synergistic induction of SAA gene by proinflammatory cytokines.

However, the SAA gene is thought to be synergistically induced by NF-κB and C/EBPβ in response to IL-1 and IL-6,
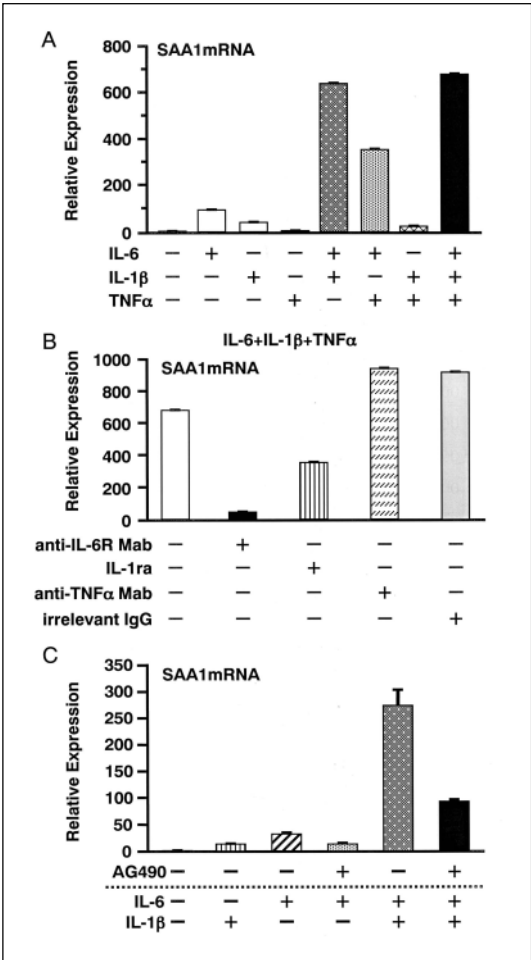

Fig.1 IL-6 plays a critical role in SAA1 mRNA induction by proinflammatory cytokines

(A) Combined effects of IL-6 (10 ng/ml), IL-1β (0.1 ng/ml), and/ or TNFα (10 ng/ml) on the induction of SAA1.

(B) Inhibitory effects of anti-IL-6R Mab (25 μg/μl), IL-1 ra (100 ng/ml), and anti-TNFα MAb (4 μg/μl) on the synergistic induction of SAA1 generated by triple stimulation with IL-6, IL-1β, and TNFα. Each specific reagent was incubated with HepG2 cells for 30 min prior to the cytokine stimulation.

(C) Effects of Jak2 kinase inhibitor-AG490 (100 μM) on synergistic induction of SAA1 mRNA. HepG2 cells were treated with AG490 or DMSO alone for 30 min before cytokine stimulation. SAA1 mRNA in HepG2 cells were measured by real-time quantitative RT-PCR at 6h after cytokine stimulation. Values represent the means + SD of duplicate measurements. The same experiment was repeated at least three times, and representative data are shown.

respectively. Since IL-6 has two main signal transduction pathways, the MAPK and Jak-STAT pathways, which regulate C/EBPβ and STAT3[8], we used Jak2 inhibitor-AG490 to deter-

mine whether the Jak-STAT pathway is involved in the expression of the SAA gene. Suprisingly, AG490 reduced the synergistic induction of SAA1 to 30%[6] (Fig.1C). These findings therefore indicate that the Jak-STAT pathway plays an important role in the synergistic induction of the SAA gene. It has further been repoted that STAT3 binds to a γ-interferon activation sequence (GAS) like sequence (-TTNNNGAA)[8]. The C-reactive protein (CRP) gene, a major acute phase protein activated by IL-6, has a STAT3 response element (RE) (-TTCCCGAA) in its promoter[9]. However, no STAT3 RE has been found in the human SAA1 promoter[4].

STAT3 is essential for the synergistic induction of human SAA genes via the NF-κB RE containing region after formation of a complex with NF-κB p65

To examine the effect of STAT3 on the SAA1 promoter activity, pEF-BOS dominant negative STAT3 Y705F (dn STAT3) or pEF-BOS wild type STAT3 (wt STAT3) was co-transfected with the pGL3-SAA1 promoter luciferase construct (-796/+24) (pGL3-SAA1) into HepG2 cells[7]. dn STAT3 completely inhibited the transcriptional activity of pGL3-SAA1 even after stimulation with IL-1+6, and wt STAT3 augmented the transcriptional activity of pGL3-SAA1 three times more than that resulting from IL-6 and IL-1+6 stimulation (Fig.2A). These results indicate that STAT3 plays an essential role in the transcriptional augmentation of SAA1 genes.

Since no typical STAT3 consensus sequence has been identified in the human SAA1 promoter[4], it became necessary to determine how STAT3 is involved in the transcriptional activation of SAA gene by using 5' deletion mutants of the SAA1 promoter. We observed that the promoter activity of SAA1 genes was partly reduced by deletion of C/EBPβ RE and completely eliminated by deletion of NF-κB RE (data not shown)[7]. To examine the possibility that STAT3 might act on the transcriptional activity of SAA via the C/EBPβ and NF-κB RE-containing regions, wt STAT3 was co-transfected with a pGL3-SAA1, pGL3-SAA1 C/EBPβ RE deleted mutant (ΔC/EBPβ RE), and a pGL3-SAA1 NF-κB RE deleted mutant (ΔNF-κB RE). As seen in Fig.2B, the co-expression of wt STAT3 increased the transcriptional activity of pGL3-SAA1 and pGL3-SAA1 ΔC/EBPβ RE almost three-fold. However, co-expression of wt STAT3 did not enhance the transcriptional activity of pGL3-SAA1 ΔNF-κB RE. These results suggest that STAT3 is involved in the transcriptional activity of SAA, most likely via the NF-κB RE containing region. In the case of rat γ fibrinogen, the CTGGGAATCCC sequence
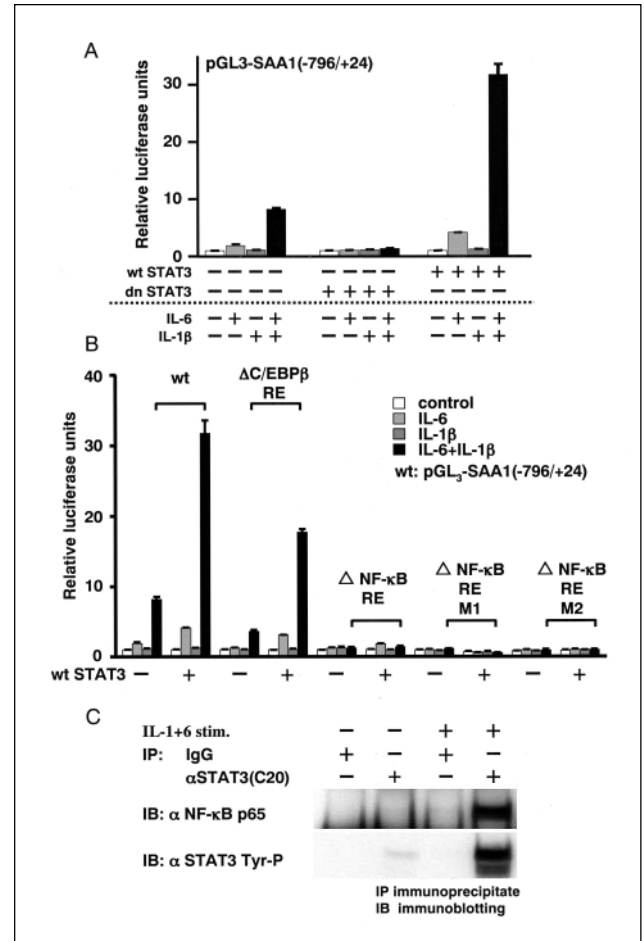

Fig.2 Essential role of STAT3 in the transcriptional activity of the SAA1 gene via the NF-κB RE containing region after formation of a complex with NF-κB p65

(A) STAT3 showed transcriptional augmentation in association with the SAA1 promoters. HepG2 cells were transfected with 0.5 μg of pGL3-SAA1 (-796/+24) alone, or in combination with 0.5 μg of pEF-BOS dn STAT3 (dn STAT3) or pEF-BOS wt STAT3 (wt STAT3), respectively.

(B) STAT3 without NF-κB RE did not enhance the transcriptional activity of SAA1. 0.5 μg of wt STAT3 was co-transfected with 0.5 μg of pGL3-SAA1 (-796/+24), pGL3-SAA1 ΔC/EBPβ RE, pGL3-SAA1 ΔNF-κB RE, pGL3-SAA1 NF-κB RE M1 (AGATCTATTTCC) or M2 (CAGGGACTTGTA). Cyokine stimulation with IL-6 (10 ng/ml) and/or IL-1β (0.1 ng/ml) was performed for 3 h. The relative luciferase activity is expressed as the means + SD of triplicate cultures and transfections.

(C) Endogeneous STAT3 interacted with NF-κB p65 following IL-1+6 treatment. Nuclear extracts of HepG2 cells stimulated with IL-1 and IL-6 were immunoprecipitated with the anti-STAT3 (C-20) antibody, and Western blots of the immunoprecipitates with anti-NF-κB p65 and anti-STAT3 antibodies were performed as shown.
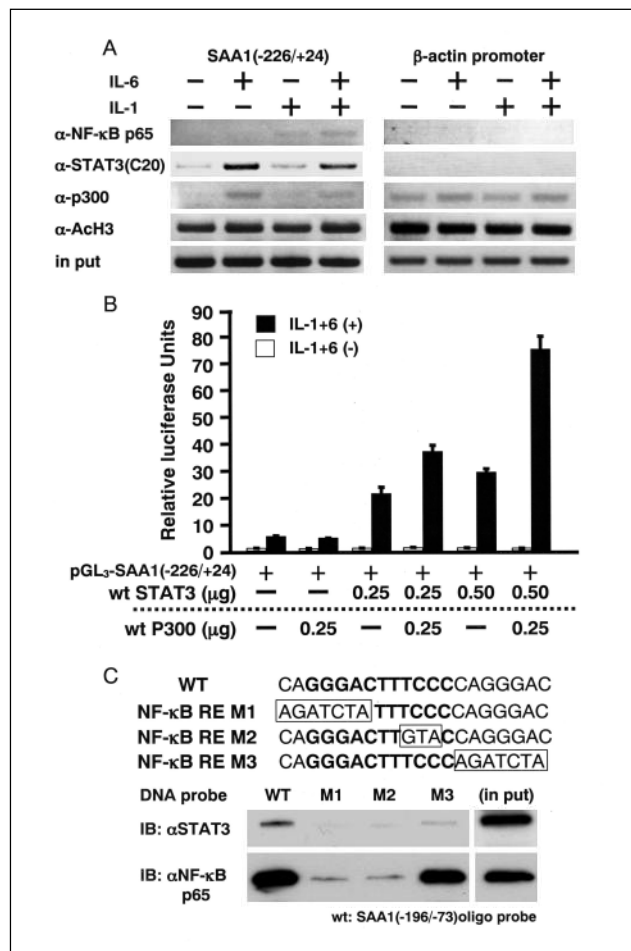
Fig.3 STAT3 acts on the SAA1 promoter by means of a newly discovered cis-acting mechanism

(A) ChIP assays demonstrate the recruitment pattern of STAT3, NF-κB p65, and p300 on the SAA1 promoter (-226/+24) from HepG2 cells treated with IL-6 (10 ng/ml) and/or IL-1β (0.1 ng/ml) for 30 min. Anti-AcH3 antibody was used as a positive control for this assay.

(B) HepG2 cells were transfected with 0.5 μg of pGL3-SAA1 (-226/+24), 0.25 μg of p300 wt in pCMV β (wt p300), and/or 0.25-0.5 μg of wt STAT3. IL-1and IL-6 stimulation was performed for 3 h. Relative luciferase activity is expressed as the means + SD of triplicate cultures and transfections.

(C) DNA affinity chromatography was performed with 200 μg of the nuclear extracts from HepG2 cells after cytokine stimulation. The nuclear extracts were mixed with 1 μg of the biotinylated DNA probe, after which 50 μl of streptavidin-Dynabeads was added to and mixed in with the samples and collected with a magnet. The trapped proteins were then analyzed by Western blotting. The SAA1 (-196/-73) mt NF-κB RE M2 and M3 probe lost their ability to interact with both STAT3 and NF-κB p65, but the SAA1 (-196/-73) mt NF-κB RE M3 probe maintains its binding affinity for NF-κB p65 but not STAT3.

was found to be responsible for transactivation by both STAT3 and NF-κB[10]. Furthermore, it has been reported that TCC was necessary for NF-κB binding and that the CTGGGAA sequence was needed for STAT3 binding because of the loss of transcriptional activity in an AGATCTATCCC mutant. To investigate whether STAT3 binds to the CAGGGAC sequence of the NF-κB RE in the SAA1 promoter region, we created two mutated constructs, pGL3-SAA1 NF-κB RE M1 (AGATCTATTTCCC) and M2 (CAGGGACTTGTAC). We expected that STAT3 would bind to NF-κB RE M2, but not to NF-κB RE M1. However, neither transfection with NF-κB RE M1 nor with NF-κB RE M2 resulted in transcriptional activity even when wt STAT3 was co-expressed (Fig.2B). These findings suggest that STAT3 does not bind to the NF-κB RE, which mediates the transcriptional activity of the human SAA1 gene. We confirmed these results with a gel shift assay using a wild type SAA1 oligonucleotide (-196/-73) probe (wt SAA1 probe) or SAA1 mutated the NF-κB RE M2 oligonucleotide (SAA1 mt NF-κB RE M2 probe) which contains the C/EBP β and NF-κB RE in the human SAA1 promoter. Two major nucleoprotein complexes were detected after cytokine stimulation using the wt SAA1 probe, but formation of the complex was diminished when the SAA1 mt NF-κB RE M2 probe was used (data not shown)[7]. This result was consistent with that for the deletion mutants of SAA1 promoter (Fig.2B) and indicated that NF-κB RE on the SAA1 promoter is essential for the transactivation of SAA1.

We assumed that STAT3 forms a complex with NF-κB and contributes to the transcriptional augmentation of the human SAA gene. To examine the validity of this hypothesis, we performed IP-Western of STAT3 and NF-κB[7]. Fig.2C clearly shows that STAT3 is associated with NF-κB p65 following IL-1+6 treatment, but that no specific band of NF-κB p50 was detected (data not shown) . These findings are consistent with those reported by Betts et al, namely, that overexpression of NF-κB p65 but not p50 enhanced the transcriptional activity of human SAA2 in a dose-dependent manner[5]. This indicates that crosstalk between STAT3 and NF-κB p65 contributes to the transcriptional augmentation of SAA by IL-1+6 stimulation.

STAT3 acts on the SAA1 promoter by means of a newly discovered cis-acting mechanism

We next investigated how STAT3 contributes to the formation of the transcritpitonal complex comprising NF-κB, C/EBP β and STAT3. STAT3 is reportedly associated with p300[11], which suggests the possibility that heteromeric complex formation of

STAT3, NF-κB p65 and p300 may be involved in the transcriptional activity of human SAA gene. To examine this possibility, we performed a Chromatin immunoprecipitation (ChIP) assay using chromatin isolated from HepG2 cells. STAT3 and p300 were strongly recruited to the SAA1 promoter region (-226 /+24) in response to IL-6 or IL-1+6 and weakly recruited by IL-1. NF-κB p65 was recruited by IL-1 or IL-1+6 and only slightly recruited so by IL-6[7] (Fig.3A). These results provide clear evidence that STAT3 forms a transcriptional complex with NF-κB p65 and p300 on the SAA promoter region. A subsequent luciferase assay using pGL3-SAA1 (-226/+24) co-transfected with p300 wt in pCMVβ (wt p300) and wt STAT3 demonstrated that co-expression of wt p300 alone did not augment the luciferase activity of pGL3-SAA1 (-226/+24), but that co-expression of wt p300 with wt STAT3 dramatically enhanced the luciferase activity in a dose-dependent manner (Fig.3B). This suggests that STAT3 interacts with p300 in the transcriptional activation of the human SAA gene.

However, the question remained how STAT3 binds to the promoter region of the SAA1 gene because no typical STAT3 RE seems to be located there. It is likely that STAT3 either binds to the promoter region of the SAA gene indirectly, or obtains binding affinity for an unknown DNA sequence in a complex with NF-κB p65. In an attempt to answer this question, we performed DNA affinity chromatography using an wt SAA1 probe. Nuclear extracts specifically interacting with a biotinylated wt SAA1 probe were collected with the aid of streptavidin-Dynabeads with a magnet and transcriptional factors were analyzed by Western blotting[7]. NF-κB p65 and STAT3 were both pulled down by the wt SAA1 probe from the nuclear extracts of HepG2 cells after IL-1+6 stimulation (Fig.3C). We next used the SAA1 mt NF-κB RE M2 probe, which has no binding affinity for NF-κB p65, to examine the possibility that NF-κB p65 has an effect on the binding affinity of STAT3 to the SAA1 promoter region. As shown in Fig.3C, neither NF-κB p65 nor STAT3 was detected by this probe, indicating that the interaction between STAT3 and NF-κB p65 is essential for the binding affinity of STAT3 to the wt SAA1 probe. We assumed that the formation of the heteromeric complex of STAT3 and NF-κB p65 gives STAT3 binding affinity to the SAA1 promoter region.

On the basis of our results and the results obtained with rat γ fibrinogen[10], we focused our attention on the 3'-site of NF-κB RE (CA*GGGACTTTCCC*CAGGGAC) as a candidate for the STAT3 binding site, because sequences contiguous to NF-κB RE may have influenced the binding affinity of STAT3. On the assumption that mutation of this site could influence the binding
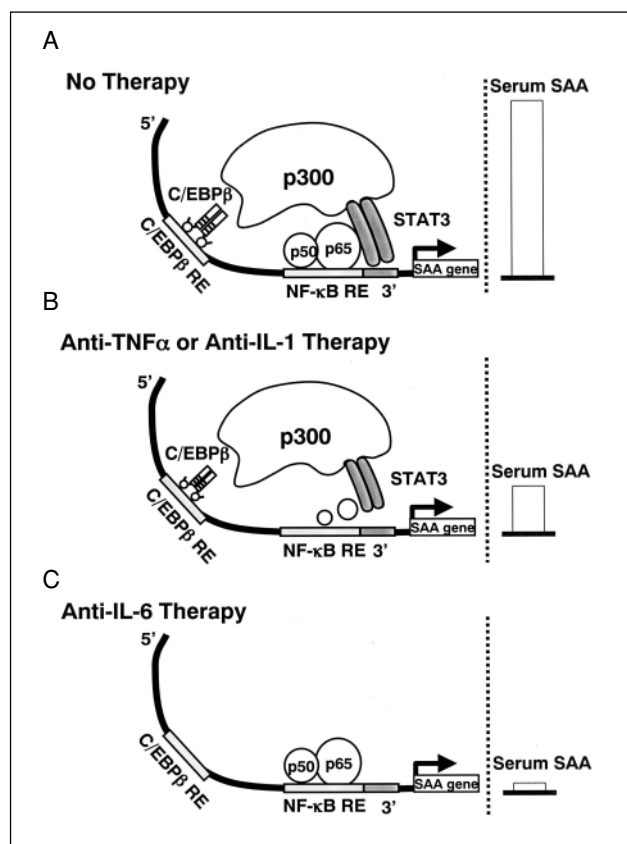

Fig.4 Effects of anti-cytokine therapy on the cytokine-driven transcriptional activity of the human SAA gene

(A) Cytokine stimulation caused the formation around the NF-κB RE of a heteromeric complex with STAT3 and NF-κB p65. STAT3, which is assumed to interact with the 3'-site of NF-κB RE, recruits the co-activator p300, which then coordinate the interaction of NF-κB p65, STAT3, and C/EBPβ thus resulting in the increased transcriptional activity of the human SAA gene.
(B) anti-TNFα or IL-1 therapy reduces the activity of the NF-κB signaling pathway, although the transcriptional complex remains.
(C) anti-IL-6 therapy inhibits the activation of STAT3 and C/EBPβ, and eliminates the formation of the transcriptional complex on the SAA promoter.

affinity of STAT3 but not of NF-κB p65, we created a SAA1 mt NF-κB RE M3 probe (CA*GGGACTTTCCC*AGATCTA). As expected, the specific bands of STAT3 from the nuclear extracts of HepG2 cells after IL-1+6 stimulation were markedly reduced by the SAA1 mt NF-κB RE M3 probe compared to the effect obtained with the wt SAA1 probe, although the specific bands of NF-κB p65 were observed almost as intact as those seen in

with the wild type (Fig.3C). These results support our hypothesis that the binding affinity of STAT3 for the human SAA promoter region is the result of the formation of a heteromeric complex comprising STAT3 and NF-κB p65.

Our overall data shows that we were able to demonstrate that STAT3 acts on the human SAA promoter via a newly discovered *cis*-acting mechanism, that is, the formation of a heteromeric complex containing STAT3, NF-κB 65 and p300. We also created a schematic model to explain the synergistic induction of the human SAA gene by IL-1+6 stimulation (Fig.4A). At the same time, this schematic model also explains the effect of anticytokine therapy on the transactivation of SAA. Anti-TNFα or IL-1 therapy reduced but did not eliminate the NF-κB signaling pathway, because this pathway is activated by various stimuli e.g. toll-like receptors, and other cytokines and so on[12]. As a consequence, the transcriptional complex on the SAA promoter persisted after Anti-TNFα or IL-1 therapy (Fig.4B). On the other hands, IL-6 family cytokines, such as oncostatin M, IL-11, and LIF, except for IL-6, have little effect on the production of acute phase protein[13]. IL-6 blocking therapy inhibits the activation of STAT3 and C/EBPβ, and also prevents the formation of the transcriptional complex on the SAA promoter (Fig.4C).

SAA is the precursor of the amyloid A protein in AA amyloidosis, and we previously observed that humanized anti-IL-6 receptor antibody therapy normalized serum levels of SAA and improved the clinical symptoms of AA amyloidosis in some Castleman's disease patients[14]. Furthermore, Gillmore et al reported that normalization of the serum SAA level can lead to amyloid regression in patients with AA amyloidsis[3]. From the clinical point of view, our findings in this context therefore constitute an aid for the development of a new therapeutic strategy for AA amyloidosis which uses normalization of the serum SAA level.

Conclusion

The clinical results of anti-IL-6 therapy for RA[2] or Castleman's disease patients[14] prompted us to investigate the exact mechanism by which proinflammatory cytokines induce SAA. This investigation enabled us to demonstrate that STAT3 plays an essential role in the synergistic induction of SAA by IL-1+6 stimulation via a new *cis*-acting mechanism. Our results further suggest that the serum SAA level is affected by the intensity of the interaction between STAT3 and NF-κB p65. NF-κB is a key regulator of pro-inflammatory cytokines, matrix metalloproteinases, inducible nitric oxide synthase, cyclooxygenase 2, and other pro-inflammatory genes[12]. The interaction between

STAT3 and NF-κB p65 may thus contribute to the expression of these pro-inflammatory genes. Our study constitutes a novel clinical research approach "from the bedside to bench", findings which are expected to aid understanding of inflammatory status and the development of a therapeutic strategy for AA amyloidosis.

References

1) Maini RN, Taylor PC: Anti-cytokine therapy for rheumatoid arthritis. Annu Rev Med, 51：207-229, 2000.

2) Nishimoto N, Yoshizaki K, Miyasaka N, Yamamoto K, Kawai S, Takeuchi T, Hashimoto J, Azuma J, Kishimoto T: Treatment of rheumatoid arthritis with humanized anti-interleukin-6 receptor antibody: a multicenter, double-blind, placebo-controlled trial. Arthritis Rheum, 50: 1761-1769, 2004.

3) Gilmore JD, Laurence BL, Persey MR, Pepys MB, Hawkins PN: Amyloid load and clinical outcome in AA amyloidosis in relation to circulating concentration of serum amyloid A protein. Lancet, 358: 24-29, 2001.

4) Uhlar CM, Whitehead AS: Serum amyloid A, the major vertebrae acute-phase reactant. FEBS, 265: 501-523, 1999.

5) Betts JC, Cheshire JK, Akira S, Kishimoto T, Woo P: The role of NF-κB and NF-IL6 transacivating factors in the synergistic activation of human serum amyloid A gene expression by Interleukin-1 and Interleukin-6. J Biol Chem, 268: 25624-25631, 1993.

6) Hagihara K, Nishikawa T, Isobe T, Sugamata Y, Song J, Yoshizaki K: IL-6 plays a critical role in the synergistic induction of human serum amyloid A (SAA) gene when stimulated with proinflammatory cytokines as analyzed with an SAA isoform real-time quantitative RT-PCR assay system. Biochem Biophys Res Commun, 314: 363-369, 2003.

7) Hagihara K, Nishikawa T, Sugamata Y, Song J, Isobe T, Taga, Yoshizaki K: Essential role of STAT3 in cytokine-driven NF-kappaB-mediated serum amyloid A gene expression. Gene Cells, 10: 1051-1063, 2005.

8) Akira S: IL-6-regulated transcription factors. Int J Biochem Cell Biol: 12, 1401-1418, 1997.

9) Zhang D, Sun M, Samols D, Kushner I: STAT3 perticipates in transcriptional activation of the C-reactive protein gene by interleukin-6. J Biol Chem, 271: 9503-9509, 1996.

10) Zhang Z, Fuller GM: Interleukin 1 β inhibits interleukin 6-mediated rat γ fibrinogen gene expression. Blood, 96: 3466, 2000.

11) Nakashima K, Yanagisawa M, Arakawa H, Kimura N, Hisatsune T, Kawabata M, Miyazono K, Taga T: Synergis-

tic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. Science, 284: 479-482, 1999.

12) Li Q, Verma IM: NF-κB regulation in the immune system. Nat Rev Immunol: 2, 725-734, 2002.

13) Feldmann M, Bennan FB, Maini RN: Role of cytokines in rheumatoid arthritis. Annu Rev Immunol, 14: 397-440, 1996.

14) Nishimoto N, Sasai M, Shima Y, Nakagawa M, Matsumoto T, Shirai T, Kishimoto T, Yoshizaki K: Improvement in Castleman's disease by humanized anti-interleukin-6 receptor antibody therapy. Blood, 95: 56-61, 2000.