An active metabolite of leflunomide, A77 1726, inhibits the production of serum amyloid A protein in human hepatocytes

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Objective. Cytokine-induced hepatic serum amyloid A (SAA) synthesis is the critical step in the pathogenesis of AA amyloidosis secondary to rheumatoid arthritis (RA). This study was conducted to provide more insight into the mechanism of SAA production in hepatocytes and its regulation.

Methods. Primary cultured normal human hepatocytes were stimulated with cytokines (IL-1β, TNF-α and IL-6) and the culture supernatants were analysed for the production of SAA. Human hepatocytes, treated or not treated with A77 1726, were stimulated with IL-1β and the cellular lysates were analysed by immunoblot using anti-phospho-specific mitogen-activated protein kinase (MAPK) and IκB-α. Acute phase-SAA (SAA1) mRNA expression was analysed by reverse transcription–polymerase chain reaction.

Results. IL-1β is a most potent inducer of SAA in normal hepatocytes. A77 1726 suppressed the production of SAA in human hepatocytes activated by IL-1β in a dose-dependent manner (0–50 μM). A77 1726 inhibited IL-1β-induced p38 and c-Jun N-terminal kinase 1/2 (JNK1/2) activation, whereas A77 1726 did not affect IL-1β-induced NF-κB activation in hepatocytes.

Conclusion. These results indicate that MAPK signalling pathways are critical in IL-1β-induced hepatic SAA synthesis. Leflunomide may suppress SAA synthesis by affecting these pathways and may therefore have some beneficial effect on AA amyloidosis secondary to RA.

Keywords: Amyloidosis, Hepatocytes, Rheumatoid arthritis, Serum amyloid A protein, Leflunomide, Mitogen-activated protein kinase.

AA amyloidosis is mainly encountered as a complication of chronic inflammatory diseases [1]. Rheumatoid arthritis (RA) is the most frequent cause of AA amyloidosis [2]. Serum amyloid A (SAA), an acute-phase reactant which is dramatically up-regulated during the inflammatory process, is produced by hepatocytes in response to proinflammatory cytokines [3]. Amyloid is thought to be 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 levels of circulating SAA are critical in the pathogenesis of AA amyloidosis [5]. It is thought that the adequate control of inflammation in the course of RA may prevent the development of AA amyloidosis [6]. In patients who have already developed AA amyloidosis, control of the primary disease may retard the progression of organ failure [7]. Although advances in the diagnosis and treatment of AA amyloidosis resulted in some benefit to patients with AA amyloidosis, understanding the molecular mechanisms of SAA synthesis and new therapeutic approaches are still required.

In humans, four SAA genes have been described. Two genes (SAA1 and SAA2) encode acute-phase SAA (A-SAA) and are coordinately induced in response to inflammation [8]. SAA3 is a pseudogene and SAA4 encodes constitutive SAA (C-SAA) [9, 10]. Proinflammatory cytokines, such as IL-1, IL-6 and TNF-α, can increase the transcription of genes for A-SAA [11]. The promoter region of the human the SAA gene contains a cytokine-responsive element that probably binds NF-κB and other cis-acting elements [12]. The regulation of SAA gene expression has been studied and characterized by different groups, and in different human hepatoma cell lines [13]. However, little is known about the molecular mechanism of cytokine-induced SAA synthesis in normal hepatocytes. This study was undertaken to clarify the mechanism by which SAA is produced in normal human hepatocytes. In this study, we showed that IL-1β promotes SAA synthesis from normal human hepatocytes and we provide evidence of the significance of the mitogen-activated protein kinase (MAPK) pathway in IL-1β-induced SAA synthesis.

Materials and methods

Reagents

A77 1726 was provided by Aventis Pharma Japan (Tokyo, Japan). Human recombinant IL-1β (1.5 x 10^8 U/mg) was kindly provided by Daunihon Chemical (Osaka, Japan). Human recombinant TNF-α and IL-6 were purchased from Genzyme (Cambridge, MA, USA). Anti-human SAA polyclonal antibodies were kindly provided by Dr N. Kubota (Eiken Chemicals, Tochigi, Japan). All other reagents were purchased from Sigma (St Louis, MO, USA).

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Cells
Human primary hepatocytes were purchased from Cell Systems (Kirkland, WA, USA). The cells were cultured in a basal medium composed of Ham’s F-12 and Leibovitz L-15 (1:1) medium (Invitrogen, Carlsbad, CA, USA), 0.2% (v/v) bovine serum albumin, 5 mM glucose (Wako Chemical, Osaka, Japan), 10^{-8} M dexamethasone (Wako) and 10^{-8} M bovine insulin (Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco, Grand Island, NY). These hepatocyte preparations were less than 0.1% reactive with CD68 monoclonal antibodies (eBioscience, San Diego, CA), indicating that these cells were free of monocytes/macrophages.

Assessment of cell viability
Cell viability was assessed using methyl thiazolyl tetrazolium (MTT) staining. Cell cultures (100 μl) of containing 1 × 10^4 cells were plated in the wells of 96-well culture plates. After incubation with A77 1726 for 24 h, 100 μl of MTT solution (2.5 mg/ml) was added. After incubation at 37°C for 4 h, 100 μl of acid isopropanol (0.04 N HCl in isopropanol) was added and mixed gently with the cell suspension, and optical density at 560 nm was determined with an enzyme-linked immunosorbent assay reader.

Immunoblot analysis
Whole-cell lysates were prepared from IL-1β-stimulated hepatocytes using Triton lysis buffer containing protease and phosphatase inhibitor (1% Nonidet P 40, 50 mM Tris, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 20 mM β-glycerophosphate, 1.0 mM sodium orthovanadate, 10 μg/ml aprotinin and 10 μg/ml leupeptin). One hundred micrograms of protein was electrophoresed on 10% sodium dodecyl sulphate–polyacrylamide gels. The fractionated proteins were transferred to nitrocellulose membranes and probed with anti-IκB-α, phospho-ERK1/2, phospho-p38 and phospho-JNK1/2 antibodies (1:1000 dilution; Biosource, San Diego, CA), indicating that these cells were free of monocytes/macrophages.

Results

Cytokine-induced SAA production in hepatocytes
To examine whether SAA can be produced from normal hepatocytes, primary cultured human hepatocytes were stimulated by cytokines. Human hepatocytes were stimulated with IL-1β, TNF-α or IL-6, and the culture supernatants were subjected to SAA immunoblot analysis. As shown in Fig. 1, the stimulation of SAA protein production was significantly induced in IL-1β-stimulated hepatocytes compared with that in hepatocytes stimulated by TNF-α or IL-6. These observations suggest that IL-1β is a key cytokine for the regulation of the magnitude of SAA production in human hepatocytes in vitro.

IL-1β-induced MAPK and NF-κB activation in hepatocytes
We then focused on the IL-1β-mediated SAA synthesis cascade in human hepatocytes. Upon binding of IL-1β to its receptor, receptor-mediated signals eventually activate MAPK and NF-κB to produce inflammatory mediators [14]. As shown in Fig. 2, IL-1β stimulation resulted in IκB-α degradation, which suggests the activation of NF-κB. IL-1β stimulation also induced the phosphorylation of MAPK (ERK1/2, p38, and JNK1/2), demonstrating the activation of the kinases (Fig. 3).

MW(KDa)

| 14.3 |
| 6.4 |

**Fig. 1.** Cytokines induce SAA synthesis from human hepatocytes. Human hepatocytes were stimulated with the indicated concentrations of cytokines for 48 h. SAA production was measured by anti-SAA immunoblot using culture supernatants. Lane 1, unstimulated; lane 2, TNF-α (20 ng/ml); lane 3, TNF-α (100 ng/ml); lane 4, IL-6 (20 ng/ml); lane 5, IL-6 (100 ng/ml); lane 6, IL-1β (51U/ml); lane 7, IL-1β (201U/ml). The data shown are representative of two independent experiments.
Inhibition of SAA induction by A77 1726 in hepatocytes

Leflunomide is an isoxazole derivative that has been proved to be efficacious in the treatment of RA. Several investigators have demonstrated that leflunomide is a potent inhibitor of MAPK and NF-κB [15, 16]. The above findings may suggest that the potent inhibitors for MAPK and NF-κB can be capable of modulating IL-1β-induced SAA synthesis in hepatocytes. We examined the effects of A77 1726, leflunomide’s active metabolite, on IL-1β-stimulated SAA production in human hepatocytes. As shown in Fig. 4, A77 1726 significantly inhibited SAA production in IL-1β-stimulated human hepatocytes at concentrations of 50 μM. The attenuating effects of A77 1726 on SAA production were not due to a toxic effect, because cell respiration analysed by MTT staining was not affected by this drug (data not shown).

To examine whether A77 1726 blocked SAA induction at the transcriptional level, we analysed A-SAA mRNA expression in human hepatocytes by RT-PCR. As shown in Fig. 5, A77 1726 modified the phosphorylation state of the MAPK activated by IL-1β.

Next, we investigated the molecular mechanism by which A77 1726 inhibited SAA synthesis in hepatocytes. We evaluated 1κB-α proteolysis. The protein levels of 1κB-α in IL-1β-stimulated quiescent hepatocytes were measured by immunoblot analysis (Fig. 6). IL-1β stimulation induced substantial 1κB-α degradation. A77 1726 treatment did not influence this 1κB-α degradation. The transcriptional activity of NF-κB was also examined. To this end, hepatocytes were transiently transfected with NF-κB-SEAP. Then, the transfectants were pretreated with A77 1726 for 12 h, a treatment which was followed by stimulation with IL-1β for 12 h. As shown in Fig. 7, no suppressive effect of A77 1726 on NF-κB-dependent transcriptional activity was observed.

We examined the effects of A77 1726 on IL-1β-induced MAPK activation in hepatocytes. Quiescent hepatocytes were incubated with 0–50 μM of A77 1726, an active metabolite of leflunomide, for 12 h. Hepatocytes pretreated with A77 1726 were then stimulated with IL-1β for 15 min. The cellular lysates were then analysed by immunoblots using anti-phospho-specific MAPK antibodies. The A77 1726 pretreatment did not affect the phosphorylation status of ERK1/2, whereas IL-1β-induced p38 and
human hepatocytes. We therefore investigated the mechanism of cytokine-induced SAA synthesis and its regulation, using normal human hepatocytes. Previous studies using primary hepatocytes, hepatoma cell lines and transfected cells have indicated that SAA synthesis can be induced by IL-6, IL-1β and TNF-α [18]. In this study, we showed that, among the cytokines studied, IL-1β is the most potent inducer of SAA protein synthesis in normal human hepatocytes. Our results are consistent with those of previous reports demonstrating that the effect of IL-1β is substantially more potent than that of the other two cytokines [19]. However, it should be noted that different human hepatoma cell lines differ in their ability to synthesize SAA in response to individual cytokines. Upon the binding of IL-1 to the IL-1 receptor (IL-1R), MyD88, an adapter protein, links to IL-1R-associated protein kinase (1RAK) and 1RAK is phosphorylated [20]. Phosphorylated 1RAK dissociates from the receptor complex and activates TNF receptor-associated factor 6 (TRAF-6) [21]. Subsequently, TRAF-6 activates MAPK and NF-κB to induce target gene expression [14].

To elucidate IL-1β signalling in SAA synthesis, we investigated MAPK and NF-κB activation in human hepatocytes. Consistent with the previous findings, our data indicated that IL-1β stimulation resulted in the activation of extracellular signal-regulated kinase 1/2, p38 and JNK1/2 in hepatocytes. NF-κB has also been shown to be activated by IL-1β stimulation, based on the rapid degradation of IκB-α observed here and elsewhere.

To extend our results further, we addressed the question of whether IL-1β-mediated SAA induction could be regulated by the modulation of these signalling pathways. The novel result is that an active metabolite of leflunomide, A77 1726, almost completely suppressed IL-1β-induced SAA protein synthesis in normal human hepatocytes. The primary mode of action of leflunomide is thought to be the inhibition of pyrimidine biosynthesis [22], but other mechanisms are involved as well [23]. Some reports have suggested that leflunomide is a potent inhibitor of NF-κB and MAPK [15, 16], which are also implicated in A-SAA gene transcription [12]. An unexpected result from the NF-κB activation study was that although the transcriptional activation of NF-κB occurred in IL-1β-stimulated hepatocytes, the inhibitory effect of A77 1726 on NF-κB was not observed. Furthermore, A77 1726 did not affect IL-1β-triggered IκB-α degradation in these human hepatocytes. Although we have not yet identified in detail the mechanism involved in the A771726-mediated suppression of SAA synthesis, several possibilities can be suggested. Recently, a novel cytokine-inducible transcription factor, designated SAA-activating factor-1 (SAF-1), was shown to be involved in A-SAA gene expression [24]. SAF-1 is a zinc finger transcription factor that is activated by many mediators, including IL-1β [24]. It was demonstrated that the MAPK signalling pathway regulated the DNA-binding activity of SAF-1 [25]. To determine whether the inhibitory effects of A77 1726 on SAA induction can be attributed to the inhibition of MAPK activation, we examined the effects of A77 1726 on MAPK in IL-1β-stimulated hepatocytes. Our results revealed that A77 1726 suppressed IL-1β-induced p38 and JNK1/2 activation. In this study, we did not identify the direct mechanism involved in leflunomide-mediated SAA suppression; however, in hepatocytes the MAPK pathway appears to be critical for IL-1β-induced SAA induction, and NF-κB may not be involved in SAA induction in normal human hepatocytes. In addition to SAF-1, CCAT enhancer binding protein (C/EBP) has been shown to play a major role in the transcriptional induction of A-SAA genes [26]. The C/EBP family is activated through the MAPK pathway [27]. It is also possible that A77 1726 inhibited IL-1β-induced SAA synthesis by affecting C/EBP.

It is thought that the adequate control of inflammation in the course of RA may prevent the development of AA amyloidosis [28]. Cytotoxic drugs, such as chlorambucil, have been thought to induce the remission of arthritis and improve the prognosis of AA amyloidosis in both juvenile and adult RA [29, 30]. However,
counselling should be undertaken before these drugs are prescribed, because of the unlicensed induction and its adverse effects. Taken together, our results suggest that new anti-rheumatic drugs suppressing MAPK pathways may provide beneficial effects in the prevention of AA amyloidosis by suppressing hepatic SAA synthesis, in addition to providing anti-inflammatory effects in RA patients.

The authors have declared no conflicts of interest.

References


