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Suppressive effect of leflunomide metabolite (A77 1726) on metalloproteinase production in IL-1β stimulated rheumatoid synovial fibroblasts

K. MIGITA*, T. MIYASHITA*, H. ISHIBASHI*, Y. MAEDA*, M. NAKAMURA*, H. YATSUHASHI*, H. IDA†, A. KAWAKAMI†, T. AOYAGI‡, Y. KAWABE‡ & K. EGUCHI† *Clinical Research Centre, National Nagasaki Medical Centre Omura, †First Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki and ‡Department of Rheumatology, National Ureshino Hospital, Ureshino, Saga, Japan

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SUMMARY

Leflunomide, an isoxazol derivative structurally unrelated to other immunomodulatory drugs, has proven to be efficacious in the treatment of rheumatoid arthritis (RA). This study was conducted to elucidate the mechanism by which leflunomide mediated antirheumatic effects. We investigated the effects of A77 1726, leflunomide's active metabolite, on mitogen-activated protein kinase (MAPK) activation in IL-1β-stimulated rheumatoid synovial fibroblasts. The effects of A77 1726 on the secretion of matrix metalloproteinases (MMPs) from rheumatoid synovial fibroblasts were also examined. A77 1726 partially suppressed IL-1β-induced ERK1/2 and p38 kinase activation. In contrast, A77 1726 efficiently suppressed IL-1β-stimulated JNK1/2 kinase activation. Although no suppressive effect was demonstrated on MMP-2, A77 1726 markedly inhibited MMP-1, 3, and 13 secretions from IL-1β-stimulated rheumatoid synovial fibroblasts. Tissue inhibitor of metalloproteinases-1 (TIMP-1) was constitutively produced from rheumatoid synovial fibroblasts and the suppressive effects of A77 1726 on TIMP-1 production were minimal. Our results suggest that the suppression of the MAPK signalling pathway and MMP synthesis in rheumatoid synovial fibroblasts is a possible mechanism for the inhibitory activity of leflunomide against rheumatoid arthritis.

Keywords leflunomide matrix metalloproteinases mitogen-activated protein kinases rheumatoid arthritis

INTRODUCTION

Leflunomide is a novel immunomodulatory agent that has recently been approved as a disease-modifying antirheumatic drug (DMARD) for the treatment of rheumatoid arthritis [1]. This isoxazole derivative is metabolized to its active form, A77 1726 [2]. The exact mechanism by which A 77 1726 exerts its effect *in vivo* is as yet unknown. Previous data suggest two possible modes of action: inhibition of dihydroorate dehydrogenase (DHODH), by which A77 1726 influences *de novo* pyrimidine biosynthesis [3] and interaction with signalling events, thereby interfering the phosphorylation of tyrosine kinases [4]. Because the immunoregulatory effects of A77 1726 occur at doses that inhibit DHODH but not tyrosine kinases [5], the interruption of *de novo* pyrimidine synthesis is thought to be the primary mode of action [6]. However, additional mechanisms of action may remain to be discovered. It has been suggested that leflunomide is a potent inhibitor of the transcription factor, activating protein-1 (AP-1) [7]. AP-1 is composed of two proteins, Fos and c-Jun [8]. c-Jun is phosphorylated by mitogen-activated protein kinases [9]. Therefore, it is possible that leflunomide has an inhibitory effect on AP-1 by affecting mitogen-activated protein kinases (MAPK). MAPK activation is thought to be involved in rheumatoid synovitis, based on the fact that MAPK is activated in rheumatoid synovium [10]. Because of the importance of MAPK in rheumatoid synovitis, we examined the effects of leflunomide on MAPK activation in rheumatoid synovial fibroblasts.

Correspondence: Dr Kiyoshi Migita, Clinical Research Center, National Nagasaki Medical Center, Kubara 2-1001-1, Omura 856–8652, Japan.

E-mail: migita@nmc.hosp.go.jp

MATERIALS AND METHODS

Reagents

A77 1726 was provided by Aventis Pharma Japan (Tokyo, Japan). Human recombinant IL-1β ($1{\cdot}5 \times 10^8$ unit/mg) was kindly provided by Dainihon Chemical Co. (Osaka, Japan). Mouse antihuman 13 antibodies were obtained from Fuji Chemical Co.

(Takaoka, Japan). Anti-phospho-specific ERK1/2, p38, JNK1/2 and antipan ERK1/2, p38, JNK1/2 antibodies were purchased from Biosource (Camarillo, CA, USA).

Isolation of synovial cells

The experimental protocol was approved by the local ethics committee, and a signed informed consent was obtained from each participant prior to commencement of the study. Synovial tissue samples were obtained from five patients with RA during synovectomy. Synovial fibroblasts were isolated from the synovial tissues by enzymatic digestion. The isolated synovial fibroblasts were used at the third or fourth passages for subsequent experiments. Synovial fibroblasts were cultured with RPMI 1640 media containing 10% FCS. For the experiment, the medium was discarded and the cells were stimulated with IL-1β (20 U/ml) in serum-free RPMI 1640, with or without A77 1726.

Assesment of cell viability

Cell viability was assessed using metyl thiazolyl tetrazolium (MTT) staining. 100 μl of cell culture containing 1×10^4 cells was 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 isoporopanol) was added and mixed gently with the cell suspension, and OD_{560} was determined with ELISA reader.

Immunoblot analysis for MAPK activation

Synovial fibroblasts were grown to subconfluence on 10 cm culture dishes and starved in a serum-free medium with various concentrations of A77 1726 for 12 h. The synovial fibroblasts were stimulated with IL-1β (20 U/ml) for 15 min. The cells were washed with cold PBS and lysed with a lysis buffer (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) for 20 min at 4°C. Insoluble material was removed by centrifugation at 15 000× g for 15 min at 4°C. The supernatant was saved and the protein concentration was determined using the Bio-Rad protein assay kit. An identical amount of protein (50 μg) for each lysate was subjected to 10% SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose membrane. The filters were blocked for 1·5 h using 5% nonfat dried milk in TBS (50 mM Tris, 0·15 M NaCl, pH 7·5) containing 0·1% Tween 20, washed in TBS and incubated at room temperature, for 2 h in a 1 : 1000 dilution of phosphospecific ERK1/2, p38 or JNK1/2 antibodies (Biosource). The filters were later washed in TBS and incubated with 1 : 1000 dilution of donkey antirabbit IgG antibodies, coupled with horseradish peroxidase. An enhanced chemiluminescence (ECL) system (Amersham, Bucks, UK) was used for detection.

ELISA

MMP-1, 2, 3, and TIMP-1 in the culture supernatants were measured by sandwich enzyme linked immunoassay (ELISA, R & D Systems Inc, USA).

Immunoblot analysis for MMPs

Culture supernatants were subjected to 8% SDS-polyacrylamide gel electrophoresis (PAGE). The fractionated proteins were transferred to nitrocellulose membranes and the filters were immunoblotted by anti-MMP-13 monoclonal antibodies (Fuji Chemical, Takaoka, Japan) at a 1 : 150 dilution.

Statistical analysis

Statistical significance was performed using Student's *t*-test.

RESULTS

A77 1726 modifies the phosphorylation state of the MAPK activated by IL-1β

Quiescent synovial fibroblasts were incubated with A77 1726, an active metabolite of leflunomide (0–100 μM) for 12 h. We checked the cell viability using MTT-staining method. These concentrations of A77 1726 had no effect on the viability of these cells (Fig. 1). Synovial fibroblasts pretreated with A77 1726 were then stimulated with IL-1β for 15 min. Cellular lysates were analysed by immunoblots using antiphospho-specific MAPK antibodies. IL-1β stimulation induces ERK1/2 phosphorylation, demonstrating activation of the kinase. A77 1726 pretreatment efficiently blocked ERK1 phosphorylation, whereas A77 1726 pretreatment showed only a moderate effect on the phosphorylation status of ERK2 (Fig. 2a). Also, IL-1β-induced p38 phosphorylation was partially inhibited by A77 1726 pretreatment (Fig. 2b). Interestingly A77 1726 pretreatment almost completely inhibited the JNK1/2 phosphorylation induced by IL-1β stimulation (Fig. 2c).

Effects of A77 1726 on MMP production

Next, we investigated the possibility that leflunomide affects MMP secretion from IL-1β-stimulated rheumatoid synovial fibroblasts. MMP-1, 2, 3, and TIMP-1 secretions were measured by ELISA method and MMP-13 secretions were analysed by immunoblot. Synovial fibroblast exhibited a significant increase in the production of MMP-1 (Fig. 3a) and MMP-3 (Fig. 3b) in response to IL-1β. A77 1726 treatment completely prevented the stimulation of synthesis of these MMPs from IL-1β-stimulated rheumatoid synovial fibroblasts. Although IL-1β slightly increased the MMP-2 secretions from rheumatoid synovial fibroblasts, A77 1726 treatment did not influence the IL-1β-induced MMP-2 production of rheumatoid synovial fibroblasts significantly (Fig. 3c). A77 1726 treatment also inhibited the MMP-13 secretion from IL-1β-stimulated rheumatoid synovial fibroblasts (Fig. 4).

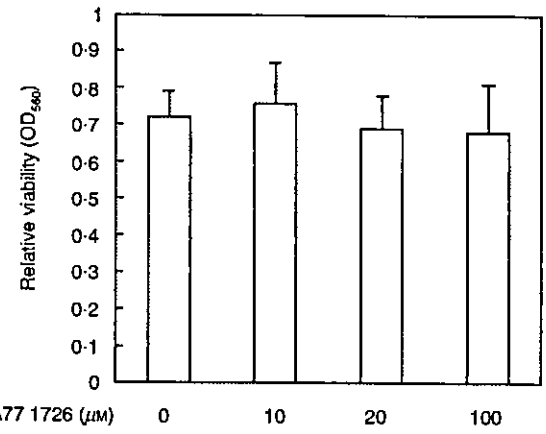

Fig. 1. Assesment of cell viability of A77 1726-treated synovial fibroblasts Synovial fibroblasts were incubated with various concentrations of A77 1726. Twelve hours later the cell viability was determined by MTT assay. Bars show the mean (SEM) of OD_{560} in three separate experiments.

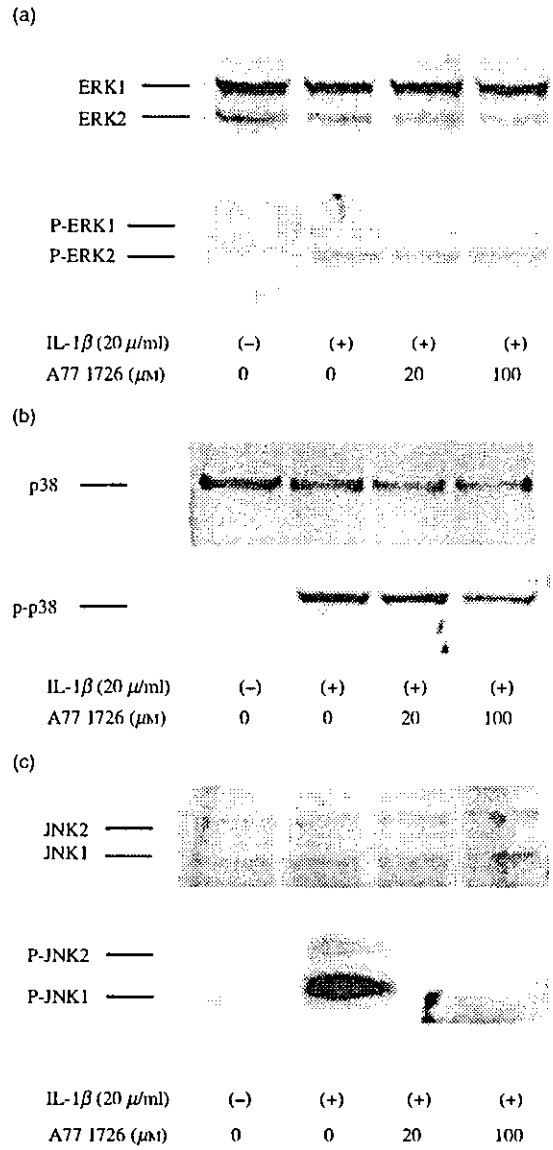

Fig. 2. Effects of A77 1726 on MAPK activation of IL-1β-stimulated rheumatoid synovial fibroblasts Rheumatoid synovial fibroblasts were pretreated with A77 1726 for 12 h in serum-free media. These quiescent synovial fibroblasts were stimulated by IL-1β for 15 min. Protein lysates from whole cell extracts were prepared as described in Materials and Methods. Protein lysates (50 μg) were subjected to 10% SDS-polyacrylamide gels, and immunoblotted using antiphospho-specific and pan-ERK1/2 (a), p38 (b), and JNK1/2 (c) antibodies. IL-1β stimulation induced the phosphorylation of MAPK (P-ERK1/2, P-p38, P-JNK1/2) in rheumatoid synovial fibroblasts. Similar results were obtained in three independent experiments using different rheumatoid synovial fibroblasts.

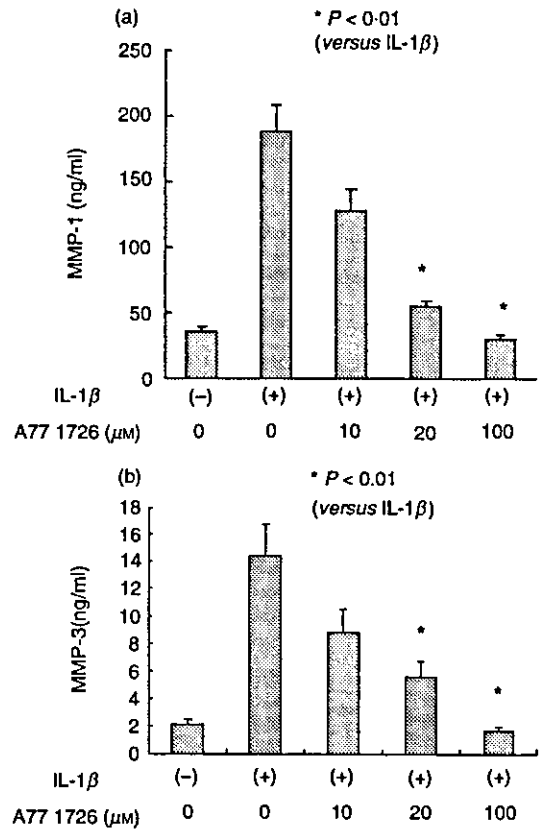

Fig. 3. Effects of A77 1726 on the MMP-1, MMP-2, and MMP-3 secretions from IL-1β stimulated rheumatoid synovial fibroblasts Rheumatoid synovial fibroblasts were stimulated by IL-1β in the presence or absence of A77 1726 for 24 h. MMP-1 (a), MMP-3 (b), and MMP-2 (c) in the culture supernatants were measured by ELISA. Bars show the mean (SEM) levels of MMPs in three separate experiments.

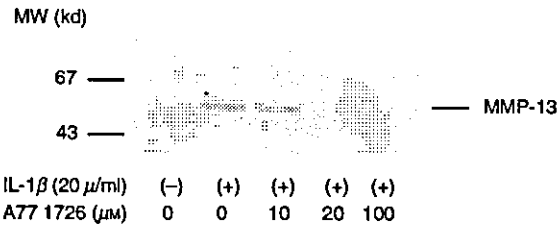

Fig. 4. Effects of A77 1726 on MMP-13 secretions from IL-1β-stimulated rheumatoid synovial fibroblasts Rheumatoid synovial fibroblasts were stimulated by IL-1β in the presence or absence of A77 1726 for 24 h. The culture supernatants were collected and subjected to electrophoresis on 10% SDS-polyacrylamide gels, and immunoblotted using anti-MMP-13 antibodies. Similar results were obtained in three independent experiments using different rheumatoid synovial fibroblasts.

Finally we investigated the effects of A77 1726 on TIMP-1 secretions. Levels of TIMP-1 secretion were high in unstimulated synovial fibroblasts and the suppressive effect of A77 1726 on TIMP-1 was not marked (Fig. 5).

DISCUSSION

It is known that leflunomide is metabolized to its active form, A77 1726, which exerts the immunosuppressive activity [2].

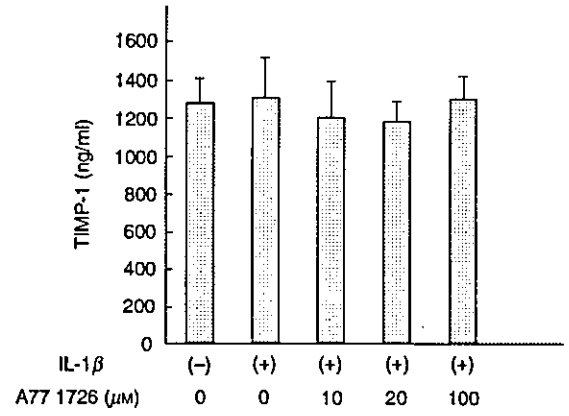

Fig. 5. Effects of A77 1726 on the TIMP-1 secretions from IL-1β stimulated rheumatoid synovial fibroblasts Rheumatoid synovial fibroblasts were stimulated by IL-1β in the presence or absence of A77 1726 for 24 h. TIMP-1 in the culture supernatants was measured by ELISA. Bars show the mean (SEM) levels of TIMP-1 in three separate experiments.

Previous data have suggested that inhibition of DHODH and interference with tyrosine kinases are two possible immunosuppressive properties of A77 1726 [2–4]. Additionally it has been suggested that leflunomide is a potent inhibitor of MAPK [7]. To determine whether the effects of leflunomide are attributable to the inhibition of MAPK activation, we examined the effects of A77 1726 on MAPK in IL-1β-stimulated synovial fibroblasts. Our results showed that leflunomide partially suppressed IL-1β-induced ERK1/2 kinase and p38 activation. In contrast, A77 1726 almost completely inhibited JNK1/2 kinase activation in the same cells. Our results clearly indicate that leflunomide is a potent inhibitor of MAPK in rheumatoid synovial fibroblasts.

Overproduction of MMP, a family of enzymes degrading the extracellular matrix components, mediates the irreversible cartilage degradation and joint destruction of RA [11]. It has been suggested that MAPK serve as the key regulator of matrix remodeling in RA, based on the fact that MAPK are activated in the rheumatoid synovium [12]. Despite the differential expression, a high degree similarity in the promoter regions of MMP exists [13]. The AP-1 site upstream of the transcriptional start site has been thought to play a critical role in the transcriptional activation of MMP gene [14]. Activation of p38 and JNK is commonly responsible for AP-1 activation through the phosphorylation of c-Jun [9]. It is conceivable that MAPK pathways are involved in coordinate AP-1 activation and MAPK inhibition is a potential therapeutic approach for the down-regulation of MMP by regulating the AP-1 transcriptional activity. Our data clearly indicate that A77 1726 prevented MMPs secretion from IL-1β-stimulated rheumatoid synovial fibroblasts. A77 1726-mediated inhibition of MAPK may decrease the MMP secretion from cytokine-stimulated rheumatoid synovial fibroblasts by affecting AP-1. Our data also indicated that A77 1726 did not influence the MMP-2 production from IL-1β-stimulated rheumatoid synovial fibroblasts. An AP-1 binding site is present within the promoter region of all of the MMP except MMP-2 [15]. It is possible that suppression of MAPK or AP-1 pathways may not be involved in the MMP-2 production from rheumatoid synovial fibroblasts.

Recent evidence suggests that A77 1726 is a potent inhibitor of nuclear factor-κB (NF-κB) activation. Manna and Aggarwal showed that 5–10 μM of A77 1726 blocks TNF-mediated NF-κB activation [7]. It is possible that A77 1726 inhibits MMPs secretion by affecting NF-κB, since this transcription factor is also involved in MMPs transcription [16]. In our experiments, the inhibitory effects on MMPs secretions were observed in the presence of >20 μM of A77 1726. This concentration of A77 1726 almost equals to the maximal plasma concentrations in RA patients with repeated oral administrations of leflunomide [1]. Previous reports also demonstrated that MMP-1 and MMP-3 secretions from rheumatoid synovial fibroblasts were inhibited at high concentrations of A 771726 concentrations (>10 μM) [17,18]. Therefore, in addition to the inhibition of NF-κB, other mechanisms, including MAPK suppression, could be involved in A77 1726-mediated MMPs suppression.

In conclusion, our data suggest that an alternative explanation for the antirheumatic efficacy of leflunomide is the potent inhibition of MAPK and MMP secretion from rheumatoid synovial fibroblasts.

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Lipopolysaccharide signaling induces serum amyloid A (SAA) synthesis in human hepatocytes in vitro

Kiyoshi Migita[a,*], Seigo Abiru[a], Minoru Nakamura[a], Atsumasa Komori[a], Yuki Yoshida[a], Terufumi Yokoyama[a], Manabu Daikoku[a], Toshihito Ueki[a], Yasushi Takii[a], Koji Yano[a], Hiroshi Yastuhashi[a], Katsumi Eguchi[b], Hiromi Ishibashi[a]

[a]*Clinical Research Center, National Nagasaki Medical Center, Kubara 2-1001-1, Omura 856-8562, Japan*
[b]*First Department of Internal Medicine, Nagasaki University School of Medicine, Japan*

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Abstract To investigate the role of lipopolysaccharide (LPS) in hepatocyte activation, we examined the expression of Toll-like receptor 4 (TLR4), the putative receptor for LPS in human hepatocytes. TLR4 mRNA and protein expression was confirmed in human hepatocytes. Stimulation of human hepatocytes with LPS results in rapid degradation of IkappaB-α and mitogen activated protein kinase activation. Human hepatocytes stimulated by LPS produced serum amyloid A protein. Our data suggest that human hepatocytes utilize components of TLR4 signal transduction pathways in response to LPS and these direct LPS-mediated effects on hepatocytes may contribute to liver inflammation and injury.

Keywords: Hepatocyte; Lipopolysaccharide; Toll-like receptor; Serum amyloid A protein

1. Introduction

Bacterial lipopolysaccharide (LPS), an essential component of the outer membrane of gram-negative bacteria, provokes a generalized proinflammatory response in the infected host that leads to septic shock and multiple organ failure [1]. LPS also causes liver injury [2]. The liver is an immunocompetent organ that plays a key role in the innate immune responses to pathogens [3]. The liver produces both inflammatory mediators and acute-phase reactants and functions to remove pathogens and microbial products from the blood [4]. Although hepatocytes have been reported to respond to LPS, the mechanisms by which LPS stimulates human hepatocytes remain uncertain. It has been suggested that Kupffer cells, as well as other macrophage populations, are responsive to LPS and produce TNF-α and IL-1 that activate hepatocytes [5]. Recently, Toll-like receptors (TLRs), several mammalian *Toll* homologues, have been identified and shown to play important roles in the recognition of various bacterial components [6]. In humans, the TLR family consists of 10 members, all of which are involved in the recognition of pathogen-associated molecular patterns [7]. Among these, Toll-like receptor 4 (TLR4) has been shown to be critical for LPS and endotoxin signaling [8]. TLR receptors have been identified on monocytes, macrophages, Kupffer cells and dendritic cells, and LPS binds to Kupffer cells via TLR4 [9]. LPS activation of Kupffer cells leads to upregulation of inflammatory cytokines, which are involved in liver damage [10]. Therefore, the general view is that the Kupffer cell is the major target of LPS in the liver.

Serum amyloid A protein (SAA) is a major acute-phase protein (APP) produced in the liver after various insults such as infection and inflammation [11]. SAA is an apolipoprotein that belongs to class 1 APP in which IL-1 and IL-6 are the main inflammatory mediators involved in its transcriptional induction [12]. Multiple *cis*-acting elements, including C/EBP and NF-κB, have been found to be important for SAA genes [13]. LPS injection in rabbit results in the activation of C/EBP and NF-κB, which may be responsible for LPS-induced SAA induction [14]. NF-κB and NF-IL6 are involved in the cytokine-induced SAA gene expression [15]. SAF-1 is a zinc finger transcription factor that is essential for cytokine-induced SAA induction [16]. It was demonstrated that MAPK signaling pathway regulated the DNA-binding activity and transactivation potential of SAF-1 and phosphorylation of SAF-1 in response to cytokines was markedly inhibited by MAPK inhibitors [17]. More recently, it was demonstrated that LPS-stimulated SAA protein induction was significantly reduced in TLR4-deificient mice, suggesting the critical role of TLR4 in SSA induction [18]. In the present study, to determine whether hepatocytes respond directly to LPS and produce SAA, we investigated the presence of TLR4, a ligand of LPS, in human hepatocytes.

2. Materials and methods

2.1. Cells

Human primary hepatocytes were purchased from Cell Systems (Kirkland, WA). The cells were cultured in a basal medium composed of Ham's F-12 and Leibovitz L-15 (1:1) medium (Invitrogen, Carlsbad, CA), 0.2% (v/v) bovine serum albumin, 5 mM glucose (Wako Chemical Co Inc., 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 hepatocytes prepara-

*Corresponding author. Fax: +81-957-54-0292.
E-mail address: migita@nmc.hosp.go.jp (K. Migita).

Abbreviations: ERK, extracellular signal-related kinase; IκB-α, IkappaB-α; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; SAA, serum amyloid A protein; TLR, Toll-like receptor

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tions were less than 0.1% reactive with the CD68 monoclonal antibodies (eBioscience, San Diego, CA), indicating that these cells were free of monocyte/macrophage.

2.2. Flow cytometry for determination of TLR4 expression

Adherent human hepatocytes were detached using 1 mM EDTA. Cell surface staining was performed using anti-human TLR4 phycoerythrin (eBioscience, San Diego, CA). Isotype-matched non-bonding control antibodies were used for comparison. Cells were analyzed using a EPCS XL (Coulter, Fullerton, CA). A total of 10 000 immunofluorescent events were acquired for each sample.

2.3. Immunoblot analysis

Whole cell lysates were prepared from LPS-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% SDS polyacrylamide gels. The fractionated proteins were transferred to nitrocellulose membranes and probed by anti-IkappaB-α (IκB-α), phospho-extracellular signal-related kinase (ERK)1/2, phospho-p38 and phospho-JNK1/2 antibodies (1:1000 dilution, Biosource, Camarillo, CA). Hepatocytes culture supernatants were also electrophoresed on 14% polyacrylamide gels. The fractionated proteins were transferred to a nitrocellulose membrane (pore-size: 0.2 μ, Bio-Rad, Hercules, CA) and probed with rabbit anti-SAA antibodies (1:2000 dilution) and developed using an enhanced chemiluminesence (ECL) system (Amersham, Arlington Heights, IL).

2.4. RNA preparation and RT-PCR assay

Total cellular RNA was extracted from hepatocytes using guanidium thiocyanate and phenol (RNAzol B, Cinna/Biotek Labs Int. Inc., Friendswood, TX). First-strand cDNA was synthesized by reverse transcription at 45 °C for 45 min in a 50 μl reaction mixture containing 1 μg of total RNA and MuLV reverse transcriptase (Invitrogen). After denaturing at 99 °C for 5 min followed by cooling at 5 °C, the cDNA was amplified using PCR. Two microliters of denatured cDNA was amplified in a 20 μl final volume containing 1 U *Taq* DNA polymerase (Gibco-BRL, Gaithersburg, MD), 1 μM of each primer, *Taq* polymerase buffer, 1.5 mM $MgCl_2$ and 1.5 mM of each dNTP. PCR was performed in a thermal cycler (Perkin–Elmer–Cetus, Foster City, CA) using a program of 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min with a final 10 min extension at 72 °C. The amplified products were subjected to electrophoresis on 2% agarose gel.

The specific primers used for TLR4 were
5′-TTGTATTCAAGGTCTGGCTGG-3′ (forward),
5′-GCAACCTTTGAAACTCAAGCC-3′ (reverse).
The predicted size of the fragment was 436 bp.
For *MyD88*:
5′-CCGCGCTGGCGGAGGAGATGGAC-3′ (forward),
5′-GCAGATGAAGGCATCGAAACGCTC-3′ (reverse).
The predicted size of the fragment was 356 bp.
For β-actin:
5′-GACGAGGCCCAGAGCAAGAGAG-3′ (forward),
5′-ACGTACATGGCTGGGGTGTTG-3′ (reverse).
The predicted size of the fragment was 236 bp.
For *SAA1*:
5′-CAGACAAATACTTCCATGCT-3′ (forward),
5′-ATTGTGTACCCTCTCCCCC-3′ (reverse).
The predicted size of the fragment was 303 bp.
For *SAA2*:
5′-CAGACAAATACTTCCATGCT-3′ (forward),
5′-ATTATATGCCATATCTCAGC-3′ (reverse).
The predicted size of the fragment was 328 bp.

3. Results

3.1. Expression of TLR4 in human hepatocytes

We first examined the mRNA expression of the LPS receptor molecule TLR4 and an intracellular adaptor protein for TLR4, *MyD88*, in human hepatocytes. Total RNA was extracted from hepatocytes and THP-1 cells, a monocyte cell line. The expression of TLR4 and *MyD88* genes was analyzed by reverse-transcription PCR. As shown in Fig. 1, TLR4 and *MyD88* mRNA were detected in human hepatocytes. THP-1 cells, which express TLR4 and *MyD88* mRNA, served as a positive control. To assess the protein expression of TLR4 in human hepatocytes, we undertook immunofluorescence staining for the presence of TLR4 in human hepatocytes using flow cytometry. As shown in Fig. 2, significant fractions of human hepatocytes constitutively expressed TLR4. To inves-

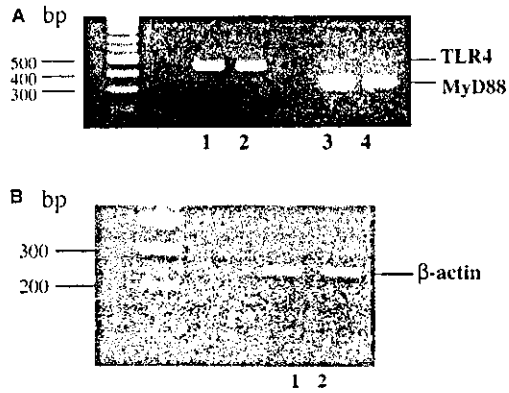

Fig. 1. Expression of TLR4 and *MyD88* mRNA in human hepatocytes. (A) Total RNA was obtained from THP-1 cells (lanes 1 and 3) and human hepatocytes and TLR4 and *MyD88* mRNA were analyzed by PCR following reverse transcription. (B) β-Actin expression in THP-1 cells (lane 1) and human hepatocytes (lane 2) were used as control.

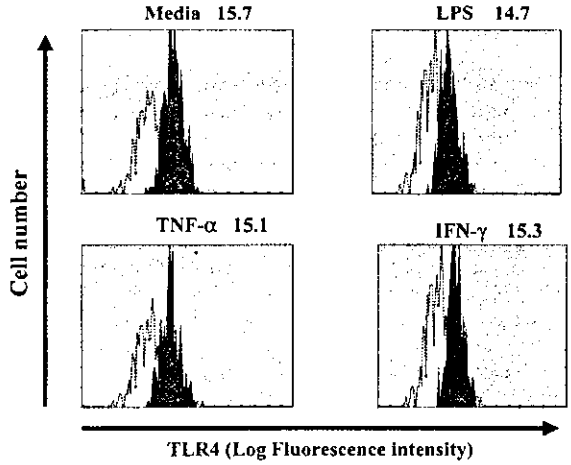

Fig. 2. Effects of cytokines on TLR4 expression on human hepatocytes. Human hepatocytes were treated with media, LPS (100 ng/ml), TNF-α (100 ng/ml) or IFN-γ(100 ng/ml) for 24 h. Expression of TLR4 on hepatocytes was measured by flowcytometer and represented as changes in fluorescence between TLR4 and the isotype control. Dotted line: isotype-matched control Ab. Solid line: TLR4 expression on hepatocytes. Values in upper right of graph represent mean fluorescence intensity of TRL4 expression. The data shown are representatives of at least three independent experiments.

tigate whether TLR4 expression could be regulated by inflammatory stimuli, we examined the effects of LPS, TNF-α and IFN-γ on TLR4 expression in hepatocytes. Human hepatocytes were stimulated with *E. coli* LPS (100 ng/ml), TNF-α (50 ng/ml) or IFN-γ (100 ng/ml) for 24 h, and the expression of TLR4 was analyzed by flow cytometry. TLR4 expression on hepatocytes was not modulated by these stimuli (Fig. 2).

3.2. LPS stimulates IκB-α degradation and MAPK activation

To determine whether LPS stimulation transmits a signal across the cell membrane in hepatocytes, we investigated the effects of LPS on the NF-κB and MAPK. Phosphorylation and subsequent degradation of IκB-α, an inhibitor of NF-κB, result in the activation of NF-κB [19]. To confirm LPS-induced NF-κB activation, we evaluated the IκB-α proteolysis. The protein levels of IκB-α in LPS-treated hepatocytes were measured by immunoblot analysis. LPS stimulation induced substantial IκB-α degradation in a time-dependent manner, suggesting the activation of NF-κB in hepatocytes (Fig. 3). LPS-mediated TLR4 signaling has also been found to trigger the activation of MAPKs. As shown in Fig. 4A, LPS had induced the phosphorylation of ERK1/2 at 15 min after stimulation. Similarly, LPS stimulation resulted in the phosphorylation of p38 (Fig. 4B) and JNK1/2 (Fig. 4C) in human hepatocytes. These results indicate that LPS activates MAPK signaling in human hepatocytes.

3.3. LPS induces SAA secretion from human hepatocytes

To investigate whether the TLR4 expressed on human hepatocytes is functional, the effects of LPS, a natural ligand for TLR4, on human hepatocytes were analyzed. We examined the secretion of SAA, a specific protein produced by hepatocytes. Human hepatocytes were incubated with varying doses of LPS in the presence or absence of 10% FCS for 24 h, and culture supernatants were removed and analyzed by anti-SAA immunoblot. Fig. 5A shows the secretion of SAA from LPS-stimulated human hepatocytes in a dose-dependent manner. LPS did not induce SAA production from human hepatocytes in the absence of serum (Fig. 5C). This result indicates that LPS-induced SAA production in hepatocytes was dependent on the presence of serum, a source of the LPS-binding protein (LBP) that is required for LPS to act through TLR4. Polymyxin B is an antibiotic that binds the lipid A motif of LPS and inactivates its biological function [20]. Preincubation with polymyxin B (100 IU/ml) before stimulation completely inhibited LPS-induced SAA production from human hepatocytes (Fig. 5B). To assess the functional role of TLR4, hepatocytes were incubated with anti-TLR4 monoclonal antibodies (HAT 125, eBioscience) for 6 h before stimulation with LPS. Hepatocytes preincubated with anti-TLR4 resulted in a reduced SAA synthesis, demonstrating that LPS utilizes TLR4 in human hepatocytes (Fig. 5D).

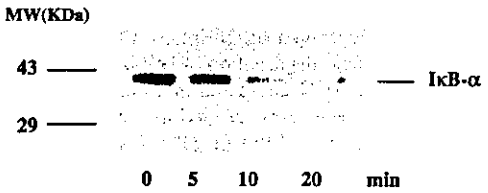

Fig. 3. LPS stimulated IκB-α degradation in hepatocytes. Human hepatocytes were stimulated with LPS (100 ng/ml) for the indicated times. Cells were lysed and cellular lysates were assessed by anti-IκB-α immunoblot analysis. The data shown are representatives of at least three independent experiments.

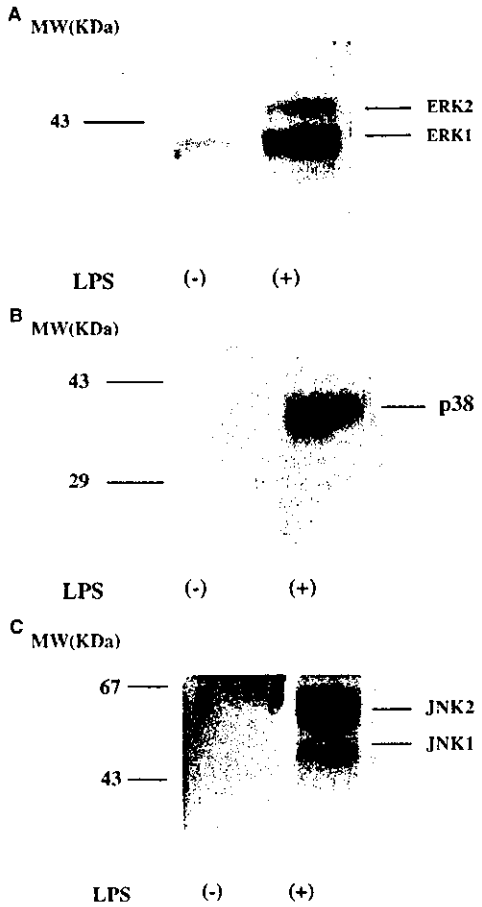

Fig. 4. LPS stimulation induces MAPK activation in hepatocytes. Quiescent human hepatocytes were stimulated with LPS (100 ng/ml) for 15 min. Cells were lysed and cellular lysates were analyzed by immunoblot using anti-phospho-specific ERK1/2 (A), p38 (B) and JNK1/2 (C) antibodies. The data shown are representatives of at least three independent experiments.

To confirm the LPS-stimulated induction of SAA at mRNA levels, we determined the levels of acute-phase SAA (*SAA1, SAA2*) in LPS-stimulated human hepatocytes using RT-PCR methods. Although the expression levels of *SAA1,2* mRNA in unstimulated hepatocytes were below the limit of detection, significant levels of *SAA1,2* mRNA expression were detected after 3 h of LPS stimulation (Fig. 6). In contrast, β-actin mRNA was constitutively expressed in hepatocytes and unchanged by LPS stimulation.

4. Discussion

Lipopolysaccharide (LPS) derived from gut bacteria has been implicated in this liver injury [21]. It has been suggested that LPS exerts an indirect effect on hepatocytes and that

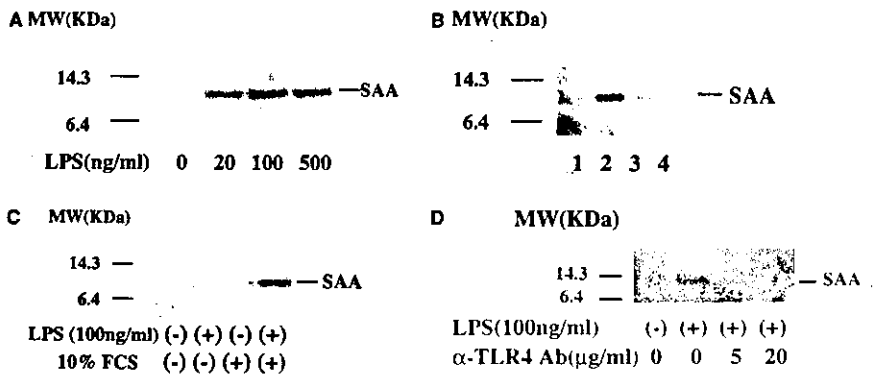

Fig. 5. (A) LPS induces SAA synthesis from human hepatocytes. Human hepatocytes were stimulated with the indicated concentrations of LPS for 24 h. SAA production was measured by anti-SAA immunoblot using culture supernatants. The data shown are representatives of at least three independent experiments. (B) LPS-induced SAA production is inhibited by Polymyxin B. Human hepatocytes were stimulated by LPS (100 ng/ml) for 24 h with or without pretreatment of polymyxin B for 30 min. SAA production was measured by anti-SAA immunoblot using culture supernatants. Lane 1: untreated; lane 2: treated with LPS (100 ng/ml); lane 3: treated with Polymyxin B (2 IU/ml) plus LPS; lane 4: treated with Polymyxin B (10 IU/ml) plus LPS. The data shown are representative of at least two independent experiments. (C) LPS-induced SAA synthesis depends on the presence of serum. Human hepatocytes were stimulated with LPS (100 ng/ml) in the presence or absence of 10% FCS for 24 h. SAA production was measured by anti-SAA immunoblot using culture supernatants. The data shown are representative of at least two independent experiments. (D) Effects of mAbs on TLR4 on the production of SAA by hepatocytes stimulated with LPS. Before the stimulation with LPS, human hepatocytes were pretreated with anti-TLR4 mAbs. SAA production was measured by anti-SAA immunoblot using culture supernatants. The data shown are representatives of at least two independent experiments.

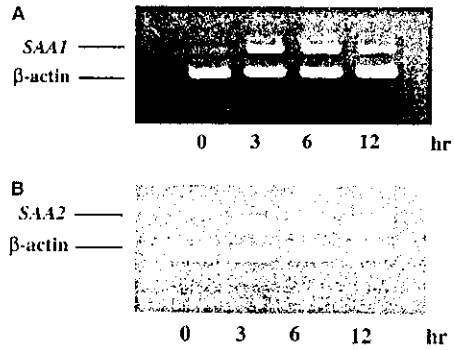

Fig. 6. LPS induces *SAA1,2* mRNA expression in human hepatocytes. Human hepatocytes were stimulated with LPS (100 ng/ml) for the indicated times. Expression on *SAA1* (A) and *SAA 2* (B) was analyzed by PCR following reverse transcription. β-Actin expression was used as control. The data shown are representatives of at least two independent experiments.

Kupffer cells are the major target of LPS [22]. However, several studies have raised the possibility that hepatocytes respond directly to LPS. Recently, it was demonstrated that murine hepatocytes express TLRs and respond to LPS through the TRL4 receptor pathway [23]. In this study, we demonstrated the mRNA expression of TLR4 and MyD88, a co-receptor for LPS, in human hepatocytes. Furthermore, hepatocytes expressed TLR4 on the cell surface at the protein level. These data indicate that human hepatocytes express LPS recognition molecules and suggest the possibility that hepatocytes respond to LPS directly.

Monocyte/macrophage exposed to LPS show the reduced surface TLR4 expression, which is thought to be one of the mechanisms of LPS tolerance [24]. In our data, TLR4 expression on hepatocytes was not modulated by LPS stimulation. Although the mechanism for the TLR4 downregulation in LPS-tolerant macrophages has not been clarified, these discrepancies in responses to LPS could be due to the differential cell types.

We then focused on the LPS signal transduction cascade in human hepatocytes. Upon binding of LPS to TLR4, MyD88, an adaptor protein, links TLR4 to IL-1R-associated protein kinase (IRAK) and IRAK is phosphorylated [25]. Phosphorylated IRAK dissociates from the receptor complex and activates tumor necrosis factor receptor-associated factor 6 (TRAF-6) [26]. Subsequently, TRAF-6 activates MAPKs and NF-κB to produce cytokines and chemokines [27]. To assess LPS signaling, we investigated whether LPS stimulation results in the activation of these kinases or transcription factors in human hepatocytes. MAPK are a group of serine/threonine protein kinases that participate in transmitting extracellular signals to the cell nucleus. LPS-induced MAPK activation was previously demonstrated in murine hepatocytes [23]. Consistent with these findings, our data indicate that LPS stimulation resulted in the activation of ERK1/2, p38 and c-Jun N-terminal kinase (JNK1/2). NF-κB is also shown to be activated by LPS stimulation. NF-κB is sequestered in an active form in the cytoplasm bound to inhibitor IκB-α. Extracellular stimuli induce IκB kinase (IKK), which degrades IκB [19]. After degradation of IκB, the NF-κB complex moves to the nucleus and activates NF-κB-dependent transcription [19]. The finding that LPS leads to the rapid degradation of IκB-α suggests that LPS stimulation induces IKK activation in human hepatocytes.

Hepatocytes support intra- as well as extrahepatic defense reactions by synthesis of APPs in inflammatory processes including endotoxemia. SAA, one of the APPs, is produced by hepatocytes in response to inflammatory cytokines such as TNF-α, IL-1β and IL-6 [11,28]. We evaluated the effects of LPS on SAA synthesis in hepatocytes. Our results clearly indicate that LPS directly induced SAA mRNA and protein expression in human hepatocytes. This LPS-induced SAA

synthesis was blocked by polymyxin B, a polypeptide antibiotic that inactivates the biological functions of LPS by removing FCS, a source of LPS-binding protein that is required for the interaction between LPS and TLR4 [29]. Recent reports indicated that NF-κB and SAA activating factor (SAF) are essential transcription factors for SAA gene expression [30] and that activation of SAF is mediated by MAPKs [17]. LPS-mediated NF-κB and MAPK activation coordinate the induction of SAA mRNA. SAA can induce chemotaxis of neutrophils [31] and matrix metalloproteinases (MMPs) induction [32], key processes involved in inflammation and tissue destructions. It is possible that human hepatocytes respond to LPS and participate in hepatic inflammation by producing SAA during LPS-induced liver injury.

In conclusion, our data suggest that human hepatocytes are capable of responding to a microbial product, LPS, directly without the mediation of Kupffer cells and macrophages. This LPS-mediated hepatocyte activation could be implicated in the pathogenesis of endotoxin-induced liver injury.

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

K. Migita[1,2,3], T. Miyashita[2], Y. Maeda[1], M. Nakamura[1,3], H. Yatsuhashi[1,3], H. Ishibashi[1,3] and K. Eguchi[1,4]

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 (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.

KEY WORDS: 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 (1.5×10^8 U/mg) was kindly provided by Dainihon 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).

[1]Clinical Research Center and [2]Department of Rheumatology, NHO Nagasaki Medical Center, Kubara 2-1001-1, Omura 856-8652, Japan, [3]Department of Hepatology, Nagasaki University School of Biomedical Science and [4]First Department of Internal Medicine, Nagasaki University School of Medicine, Sakamoto 1-7-1, Nagasaki 852-8501, Japan.

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Correspondence to: K. Migita, Clinical Research Center, NHO Nagasaki Medical Center, Kubara 2-1001-1, Omura 856-8652, Japan. E-mail: migita@nmc.hosp.go.jp

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 by anti-IκB-α, phospho-ERK1/2, phospho-p38 and phospho-JNK1/2 antibodies (JNK1/2 is c-Jun N-terminal kinase) (1:1000 dilution; Biosource, Camarillo, CA, USA). Hepatocyte culture supernatants were also electrophoresed on 14% polyacrylamide gels. The fractionated proteins were transferred to a nitrocellulose membrane (pore size 0.2 μ; Bio-Rad, Hercules, CA, USA) and probed with rabbit anti-SAA antibodies (1:2000 dilution) and developed using an enhanced chemiluminescence system (Amersham, Arlington Heights, IL, USA).

RNA preparation and RT-PCR assay

Total cellular RNA was extracted from hepatocytes using guanidium thiocyanate and phenol (RNAzol B; Cinna/Biotek Labs, Friendswood, TX). First-strand cDNA was synthesized by reverse transcription (RT) at 45°C for 45 min in a 50 μl reaction mixture containing 1 μg of total RNA and MuLV reverse transcriptase (Invitrogen). After denaturing at 99°C for 5 min followed by cooling at 5°C, the cDNA was amplified using the polymerase chain reaction (PCR). Two microlitres of denatured cDNA was amplified in a 20 μl final volume containing 1 U *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD, USA), 1 μM of each primer, *Taq* polymerase buffer, 1.5 mM $MgCl_2$ and 1.5 mM of each dNTP. PCR was performed in a thermal cycler (Perkin-Elmer-Cetus, Foster City, CA, USA) using a program of 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min with a final 10 min extension at 72°C. The amplified products were subjected to electrophoresis on 2% agarose gel.

The specific primers used were as follows: for *SAA1*, 5'-CAGACAAATACTTCCATGCT-3' (forward) and 5'-ATTGTG TACCCTCTCCCCC-3' (reverse). The predicted size of the fragment was 303 bp; for β-actin, 5'-GACGAGGCCCAGAGC AAGAGAG-3' (forward) and 5'-ACGTACATGGCTGGGGTG TTG-3' (reverse). The predicted size of the fragment was 236 bp.

Plasmids and transfection

pNFκB-SEAP (Clontech Laboratories, Palo Alto, CA, USA) was designed to monitor the activation of NFκB and NFκB-mediated signal transduction pathways. pNFκB-SEAP contained four tandem copies of the κB4 enhancer fused to the HSV-TK promoter. pTAL-SEAP (Clontech Laboratories) was used as a negative control to determine the background signals associated with the culture medium. Cells were grown to approximately 50% confluence on 30-mm plates. Transfections were done using calcium phosphate regents and following the manufacturer's instructions (Clontech Laboratories), and the cells were treated as described above. Chemiluminescence detection of the SEAP activity was performed according to the manufacture's instructions (Clontech Laboratories) using a plate fluorometer (Berthold, Bad Wildbach, Germany).

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 anti-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

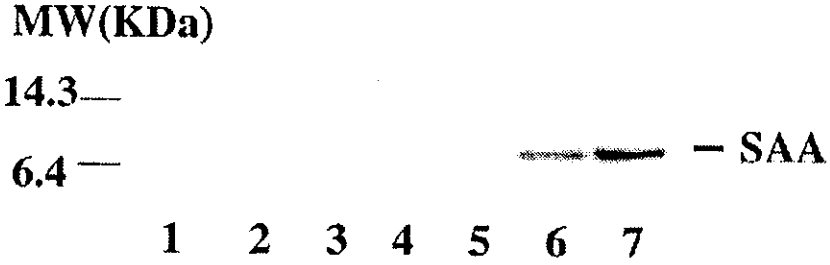

FIG. 1. Cytokines induce SAA synthesis from human hepatocytes. Human hepatocytes were stimulated with the indicated concentrations of cytokines for 48 h. SAA production was measured by anti-SAA immunoblot using culture supernatants. Lane 1, unstimulated; lane 2, TNF-α (20 ng/ml); lane 3, TNF-α (100 ng/ml);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

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).

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 patent 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 10–50 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, *SAA1* mRNA expression was markedly induced in IL-1β-stimulated human hepatocytes. This IL-1β-stimulated *SAA1* mRNA expression was inhibited by 50 μM of A77 1726.

A77 1726 modifies 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 IκB-α proteolysis. The protein levels of IκB-α in IL-1β-stimulated quiescent hepatocytes were measured by immunoblot analysis (Fig. 7). IL-1β stimulation induced substantial IκB-α degradation.

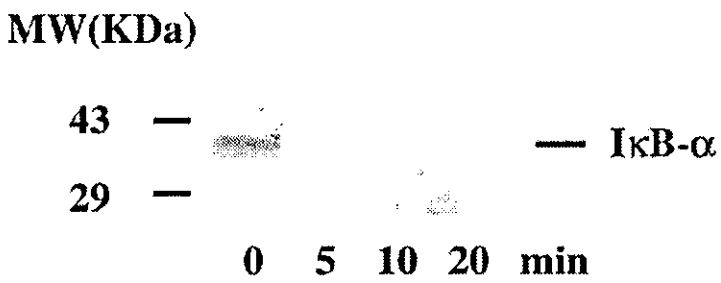

FIG. 2. IL-1β stimulated IκB-α degradation in hepatocytes. Human hepatocytes were stimulated with IL-1β (20 IU/ml) for the indicated times. Cells were lysed and cellular lysates were assessed by anti-IκB-α immunoblot analysis. The data shown are representative of two independent experiments.

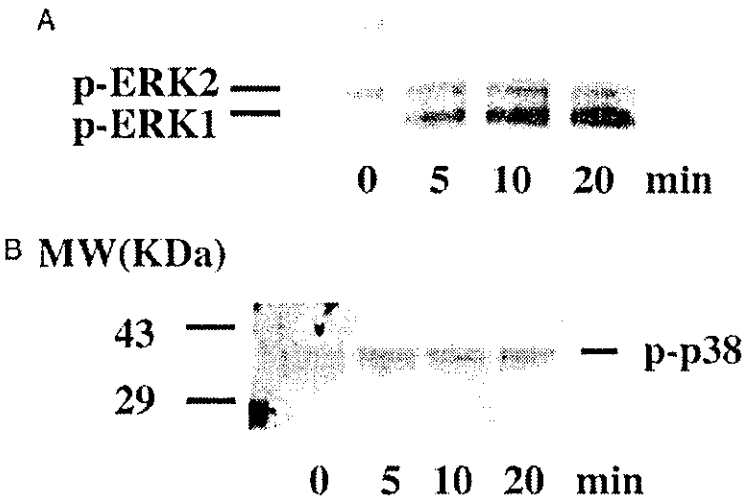

FIG. 3. IL-1β stimulation induces MAPK activation in hepatocytes. Quiescent human hepatocytes were stimulated with IL-1β (20 IU/ml) for 15 min. Cells were lysed and cellular lysates were analysed by immunoblot using anti-phospho-specific ERK1/2 (A), p38 (B) and JNK1/2 (C) antibodies. The data shown are representative of three independent experiments.

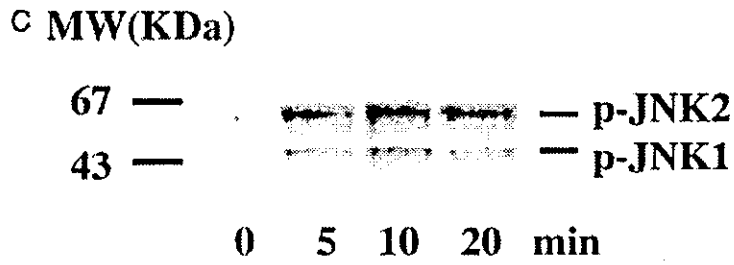

FIG. 3. Continued.

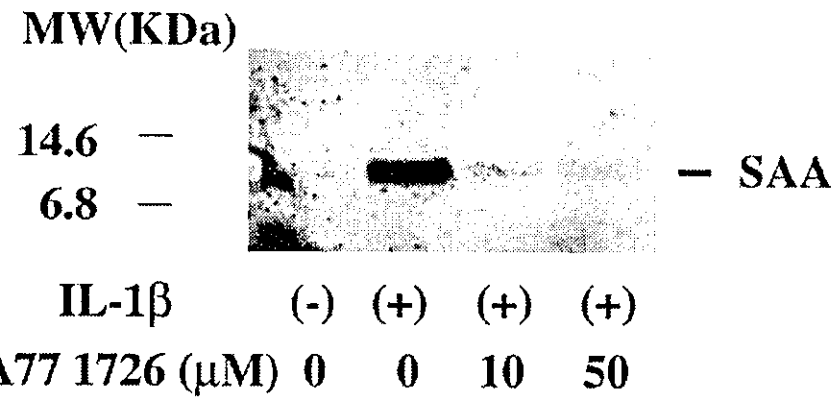

FIG. 4. SAA synthesis from hepatocytes is inhibited by A77 1726. Human hepatocytes were stimulated by IL-1β (20 IU/ml) for 48 h with or without A77 1726. SAA production was measured by anti-SAA immunoblot using culture supernatants. The data shown are representative of three independent experiments.

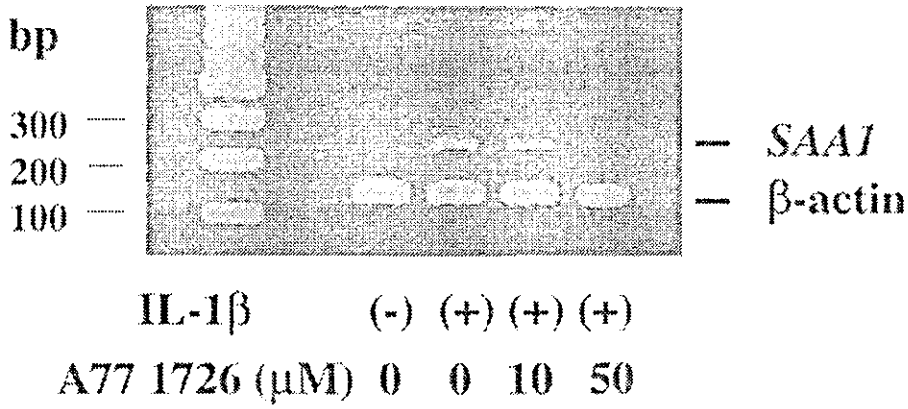

FIG. 5. IL-1β-induced *SAA1* mRNA expression in human hepatocytes was blocked by A77 1726. Human hepatocytes were stimulated with IL-1β (20 IU/ml) with or without A77 1726. *SAA1* mRNA expression was analysed by PCR following reverse transcription. β-Actin expression was used as control. The data shown are representative of three independent experiments.

A77 1726 treatment did not influence this IκB-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

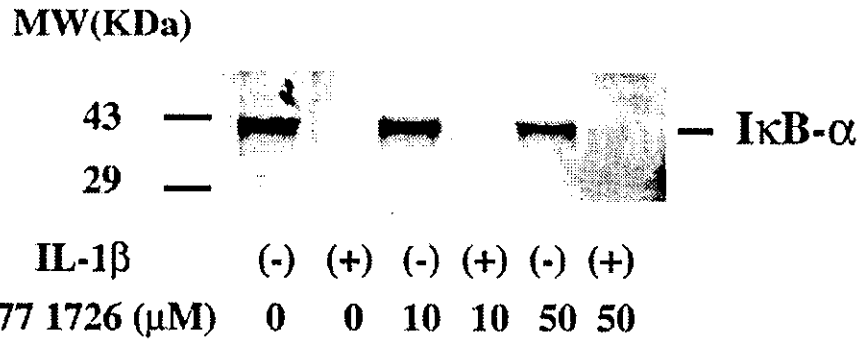

FIG. 6. Effects of A77 1726 on IL-1β-induced IκB-α degradation in hepatocytes. Human hepatocytes were pretreated with A77 1726 then stimulated with IL-1β (20 IU/ml) for 20 min. Cells were lysed and cellular lysates were assessed by anti-IκB-α immunoblot analysis. The data shown are representative of three independent experiments.

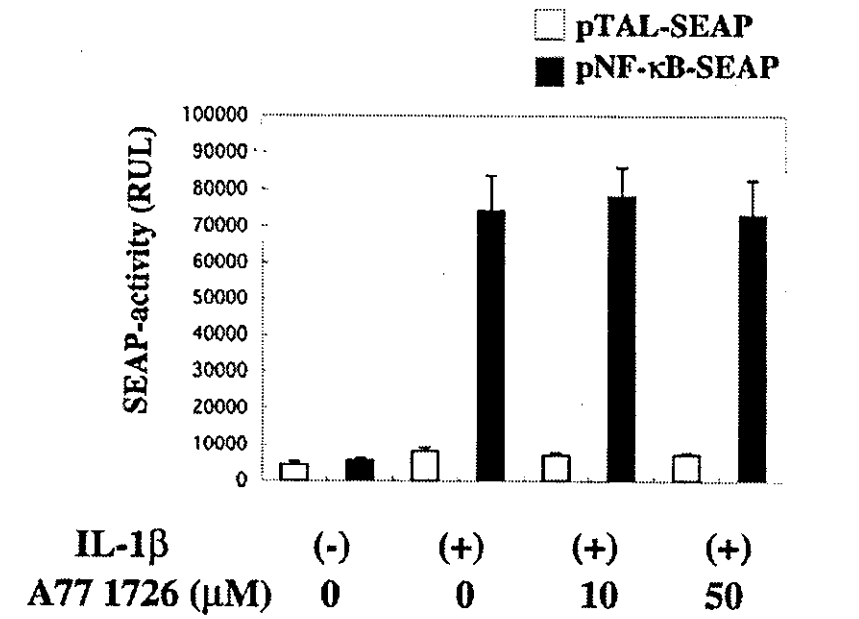

FIG. 7. IL-1β-induced SEAP expression of hepatocytes transfected with pNFκB-SEAP. Human hepatocytes were transfected with pNFκB-SEAP and a control plasmid, pTAL-SEAP and stimulated with IL-1β (20 IU/ml) with or without A77 1726 pretreatment. A significant increase in SEAP expression was observed in pNFκB-SEAP-transfected and IL-1β-stimulated hepatocytes. Columns represent mean ± S.E. of data obtained from three independent and duplicate experiments.

JNK1/2 phosphorylation was inhibited by pretreatment with A77 1726 (Fig. 8).

Discussion

The overexpression of SAA during chronic inflammation is linked to the pathophysiology of RA and AA amyloidosis [1]. The prognosis of RA patients with AA amyloidosis is still poor and the 50% survival rate ranges between 2 and 4 yr [17]. Circulating SAA is the precursor of AA amyloid deposited in the tissue [4]. Reduction of the amount of precursor protein, which arrests further deposition of amyloid, is currently the most rational approach for the management of AA amyloidosis [6]. Normalization of SAA to the baseline values can be achieved by the complete abolishment of hepatic SAA synthesis. Because of its link with AA amyloidosis, the mechanism of SAA induction has been the subject of intensive investigation. However, only limited data are available on the production of SAA in normal 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.

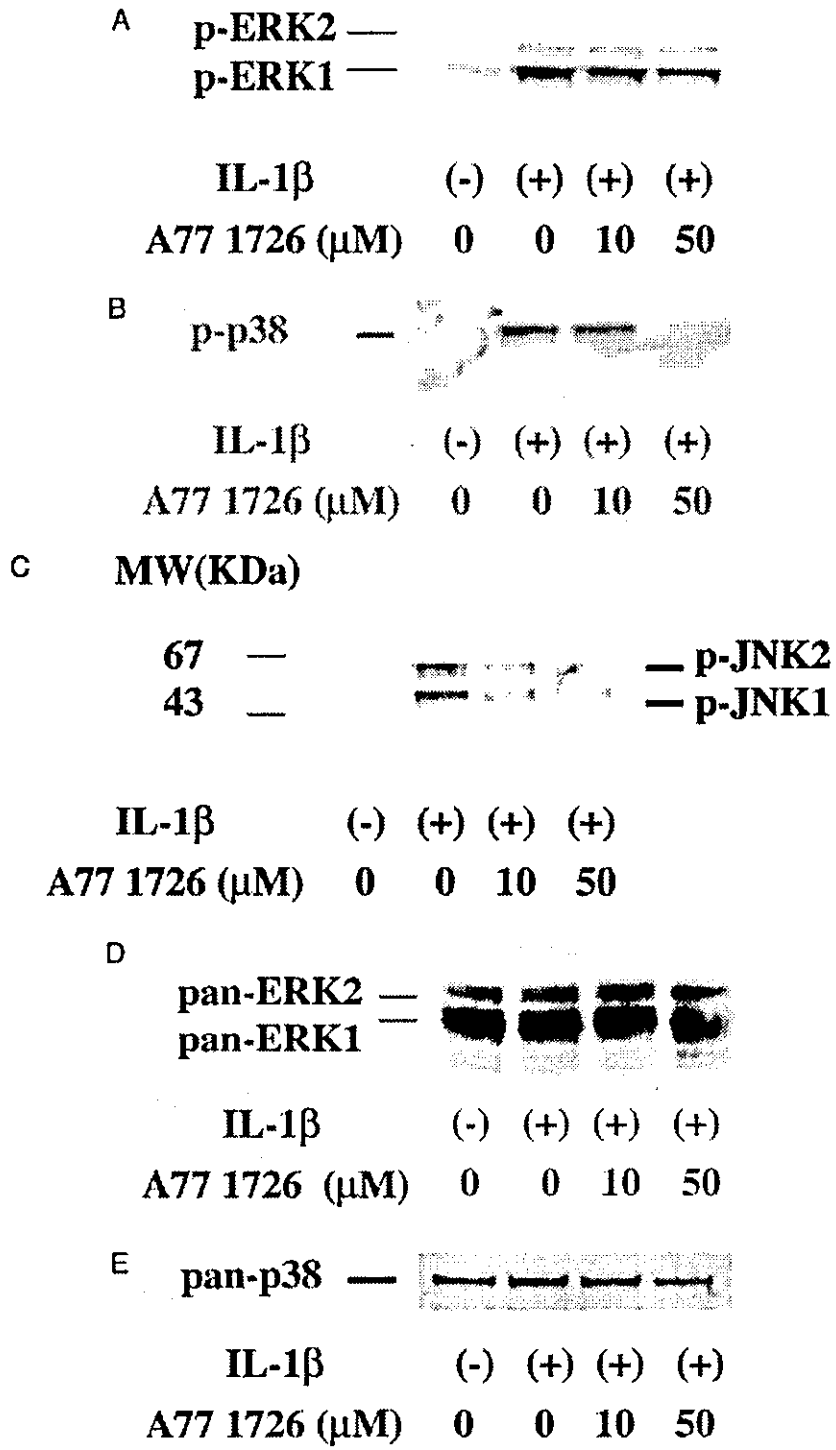

FIG. 8. Effects of A771726 on MAPK activation of IL-1β-stimulated hepatocytes. Hepatocytes were pretreated with A771726 for 12 h in serum-free media. These quiescent cells were stimulated with IL-1β (20 IU/ml) for 20 min. Protein lysates from whole cell extracts were prepared as described in Materials and methods. Protein lysates (50 μg) were subjected to 10% SDS–polyacrylamide gels and immunoblotted using anti-phospho-specific ERK1/2 (A), p38 (B), JNK1/2 (C) and anti-pan ERK1/2 (D), p38 (E), JNK1/2 (F) antibodies. Similar results were obtained in three independent experiments.

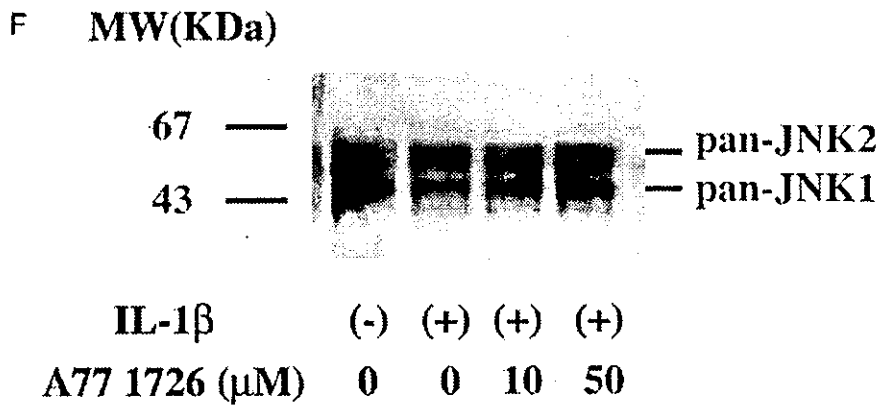

FIG. 8. Continued.

Upon the binding of IL-1 to the IL-1 receptor (IL-1R), MyD88, an adapter protein, links to IL-1R-associated protein kinase (IRAK) and IRAK is phosphorylated [20]. Phosphorylated IRAK 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-related 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. 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, CCAAT 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.

Rheumatology	Key messages
	• An active metabolite of leflunomide, A77 1726, inhibits cytokine-stimulated mitogen-activated protein kinases (MAPK) and suppresses the SAA synthesis from human hepatocytes. • Activation of MAPK is critical for hepatic SAA synthesis.

The authors have declared no conflicts of interest.

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Liver Assist Devices

Influence of serum from rats with fulminant hepatic failure on hepatocytes in a bioartificial liver system

Y. ITO, S. EGUCHI, Y. KAMOHARA, H. INUO, K. YAMANOUCHI, S. OKUDAIRA, K. YANAGA, J. FURUI, T. KANEMATSU

Department of Transplantation and Digestive Surgery, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki - Japan

ABSTRACT: Fulminant hepatic failure (FHF) is a life-threatening condition marked by many excessively increased unmetabolized toxins and growth factors. Recently developed bioartificial liver (BAL) systems containing hepatocytes can be used to treat patients with FHF. However, the behavior of these hepatocytes on exposure to FHF serum in vitro *remains unclear. In the present study, we used FHF rat models and the sera from these rats (i.e., FHF serum) contained elevated inflammatory cytokines (TNF-α, IL-1ß, and IL-6), HGF, and TGF-ß1. In addition, 1x10⁸ hepatocytes were harvested from the livers of inbred rats and incubated with microcarrier beads. Four hours later, the hepatocyte-coated beads were inoculated into a hollow-fiber module (=BAL system). FHF serum or normal control serum circulated for 6 hours through the BAL system. Expressions of mRNA for albumin, GST A1, CYP 1A2, OTC and c-fos were investigated by RT-PCR, and PCNA staining was performed before and after perfusion. The expressions of albumin, GST A1, and CYP 1A2 mRNAs were markedly decreased, whereas those of OTC and c-fos were modestly decreased. PCNA positive cells were low and showed no difference between FHF and normal serum-exposed hepatocytes. In conclusion, the exposure of hepatocytes to hypercytokinemia, including inflammatory cytokines and positive and negative growth factors, caused a loss in liver specific functions. This environment also failed to facilitate hepatocyte regeneration. (Int J Artif Organs 2004; 27: 303-10)*

KEY WORDS: Fulminant hepatic failure, Hepatocyte, mRNA, Cytokine, Rat

INTRODUCTION

Fulminant hepatic failure (FHF) is a life-threatening condition that stems from the loss of metabolic functions. Patients are exposed to various hepatotoxic substances, resulting in coagulopathy, encephalopathy, cerebral edema, and multiple organ failure (1).

Orthotopic liver transplantation has become the established therapy for FHF; however, owing to organ shortage and rapid progression of liver failure, many people lose the chance to recover (2, 3).

Over the last decade, several bioartificial liver (BAL) support systems have been developed for the purpose of bridging patients with FHF to liver transplantation or to regeneration of the diseased liver. These systems utilize viable hepatocytes from humans, human cell lines, rabbits and pigs (4-10). Some of them have been clinically tested and proved to have some efficacy (8-10). The inoculated hepatocytes seem to be active metabolically and to perform detoxification in a circuit. Nevertheless the effect of FHF serum on hepatocyte function remains unclear.

It is well known that FHF serum contains many regulating factors such as inflammatory cytokines (TNF-α, IL-1ß, and IL-6), hepatocyte growth factor (HGF), and transforming growth factor-ß1 (TGF-ß1) as well as toxins (11-14). During clinical treatment, hepatocytes in the bioreactor are directly or indirectly exposed to FHF serum. Although several *in vitro* studies on interaction between FHF serum and hepatocytes have been reported, their results are not consistent (15-19). Moreover, only a few studies have investigated effects on hepatocytes in a BAL system (20).

In the present study, we evaluated rat hepatocyte changes in response to perfusion with FHF serum, focusing on liver gene expression.

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