expression (Gracie et al., 1999). In the present study, we investigated the role of IL-18 in rheumatoid synovitis. We demonstrated that the proinflammatory cytokine IL-18 and SAA are markedly elevated in the synovial fluids of RA patients but not in those of OA. Furthermore, we examined the effects of IL-18 on A-SAA mRNA in rheumatoid synovial cells and demonstrated that A-SAA mRNA induction and SAA protein synthesis was confirmed in IL-18-treated RA synovial cells.

Although the major site of SAA production during inflammation is the liver, SAA is produced by vascular smooth muscle cells, and synovial cells (Meek et al., 1994; Kumon et al., 1999) . Recent reports suggest a new role of SAA in the development of inflammation. SAA can induce chemotaxis of neutrophils and matrix metalloproteinases (MMPs) induction, a key process involved in tissue destruction that occurs in rheumatoid cartilage (Xu et al., 1995; Brinckerhoff et al., 1989). We previously reported that SAA can induce matrix metalloproteinases, an important proteinase for rheumatoid joint destruction, from synovial cells (Migita et al., 1998). These evidences indicate that IL-18-mediated SAA synthesis could be implicated in chronic inflammatory diseases. IL-18-mediated SAA synthesis in rheumatoid synovium could be involved in the synovial inflammations through the synthesis of cartilage-degrading matrix metalloproteinases. More recently, Maury et al. (Maury et al., 2000) measured the circulating levels of IL-18 in patients having RA with or without amyloidosis and they showed the levels of IL-18 were significantly higher in patients with amyloidosis than in those without amyloidosis. Taken together, our data may suggest that IL-18 may be involved in amyloidogenesis through the perpetuation of rheumatoid synovitis as well as the production of amyloid precursor protein, SAA during inflammatory process.

In conclusion, we demonstrated for the first time the association between IL-18 and SAA in rheumatoid synovial fluids. Furthermore, IL-18 induced SAA synthesis from RA synovial cells. These data support the concept of IL-18 as an inducer of proinflammatory SAA and suggest a possible role of IL-18 in amyloidogenesis.

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Erythromycin Suppresses the Expression of Cyclooxygenase-2 in Rheumatoid Synovial Cells

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ABSTRACT. ***Objective.*** To investigate whether erythromycin (EM) can suppress the expression of cyclooxygenase-2 (COX-2) in rheumatoid synovial cells, and determine the mechanisms involved.

Methods. Synovial tissues were obtained from 25 patients with rheumatoid arthritis (RA). Rheumatoid synovial cells were cultured with or without EM (0.1–1000 nM) in the presence of interleukin 1ß (IL-1ß) for various times. Protein expression of COX-2, and phosphorylation of extracellular signal regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (p38 MAPK) were detected by Western blot. COX-2 messenger RNA (mRNA) was detected by RT-PCR. DNA binding activity of nuclear factor kappa B (NF-κB) was detected by ELISA.

Results. IL-1ß-stimulated synovial cells expressed COX-2 protein. EM suppressed the IL-1ß-induced COX-2 protein expression in a dose-dependent manner and inhibited IL-1ß-induced p38 MAPK phosphorylation, which was correlated with COX-2 expression in synovial cells. In contrast, EM had no effect on DNA binding activity of NF-κB and ERK1/2 expression.

Conclusion. Our results indicated that EM downregulated COX-2 expression by inhibiting the p38 MAPK cascade, but had no effect on NF-κB or ERK1/2, in rheumatoid synovial cells. (J Rheumatol 2004;31:436–41)

Key Indexing Terms:
ERYTHROMYCIN SYNOVIAL CELLS CYCLOOXYGENASE-2
INTERLEUKIN 1ß P38 MITOGEN ACTIVATED PROTEIN KINASE

Rheumatoid arthritis (RA) is characterized by cytokine production from inflammatory cells that infiltrate the synovial tissue and subsequent articular cartilage destruction[1]. Prostaglandin E_2 (PGE_2), an important inflammatory mediator, is produced through cyclooxygenase (COX) enzymes from prostaglandin endoperoxides[2,3]. COX-2 is an important therapeutic target in arthritis, because PGE_2 induces tissue degradation and bone resorption[4-7]. In addition, high expression levels of COX were observed in RA synovia compared with those from patients with osteoarthritis or healthy subjects[8].

Fourteen-member ring macrolides, including erythromycin (EM), exhibit antibacterial activity and a broad spectrum of pharmacological effects including antiinflammatory activity[9,10]. The macrolides act on several pathways of the inflammatory process, such as the migration of neutrophils, oxidative burst in phagocytes, and production of proinflammatory cytokines[11-13]. Previous studies showed that EM suppresses the inflammatory response by reducing PGE_2 synthesis in peritonitis[9]; however, this effect is not clear in RA. The precise mechanisms of macrolide-mediated PGE_2 synthesis inhibition remain to be clarified.

We investigated the effect of EM on COX-2 expression in rheumatoid synoviocytes. Our results showed that EM suppresses interleukin 1ß (IL-1ß)-mediated COX-2 messenger RNA (mRNA) and protein expression. We also examined the effect of EM on the mitogen-activated protein kinase cascades involved in COX-2 induction.

MATERIALS AND METHODS

Reagents. EM made by Sigma Chemical Co. (St. Louis, MO, USA) was provided from Dai-nippon Pharmaceutical (Tokyo, Japan). Human recombinant IL-1ß was purchased from Becton Dickinson Labware (San Jose, CA, USA), anti-human COX-2 rabbit IgG monoclonal antibody from IBL Corporation (Gunma, Japan), anti-rabbit IgG horseradish peroxidase (HRP) conjugate from Promega Corporation (Madison, WI, USA), rabbit anti-phospho-extracellular signal regulated kinase 1/2 (ERK1/2) and rabbit anti-phospho-p38 mitogen-activated protein kinase (p38 MAPK) from BioSource International Inc. (Camarillo, CA, USA).

Cell culture. The experimental protocol was approved by the ethics committee of Kurume University, and a signed informed consent was obtained from each participant. Synovial tissue samples were obtained from patients with RA during surgery. Synovial membranes were minced aseptically and then dissociated enzymatically with collagenase (4.0

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mg/ml; Sigma) in RPMI 1640 for 2 h at 37°C. The obtained cells were plated on culture dishes and allowed to adhere. To eliminate nonadherent cells from synovial cells and to simplify synovial B cells, the plated cells were cultured 18 h with RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37°C aerated with humidified 5% CO_2 in air. The cells were then washed thoroughly with phosphate buffered saline (PBS) solution. Adherent synovial cells were removed by adding trypsin-EDTA followed by washing the cells with PBS containing 2% FBS. The collected synovial cells were used at the second or fourth passage for subsequent experiments. Synovial cell preparations at the fourth passage contained less than 1% cells reactive to monoclonal antibodies CD3, CD20, and CD68 (Coulter Immunology, Hialeah, FL, USA) and anti-human von Willebrand factor (Immunotech, Marseilles, France), indicating that these preparations were almost free of mature T lymphocytes, B lymphocytes, monocytes/macrophages, and vascular endothelial cells. Synovial cell preparations at the second passage contained the same cells at slightly greater proportions than at fourth passage.

Immunoblot analysis. COX-2 protein expression in synovial cells was analyzed by Western blot as described[14]. For this purpose, second-passage cells were grown to subconfluence on culture dishes in serum-free medium for 24 h in the presence or absence of EM. Fourth-passage cells were grown to subconfluence on culture dishes containing serum-free medium for 1 h in the presence or absence of EM, and these cells were stimulated with IL-1ß (0.1 ng/ml) for 24 h. Cells were washed with cold PBS and lysed by the addition of a lysis buffer containing 1% Nonidet P-40, 50 mM Tris (pH 7.5), 100 mM NaCl, 5 mM EDTA, 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 supernataht was saved, and the protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). An identical amount of protein (30 µg) from each lysate and culture supernatant was subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Phosphorylation of ERK1/2 and p38 was analyzed by Western blot using phospho-specific antibody. Cells were grown to subconfluence on culture dishes, and starved by serum-free medium for 24 h in the presence or absence of EM. After starvation, synovial cells were stimulated with IL-1ß (0.1 ng/ml) for 10 min. Cells were washed with cold PBS and lysed by the addition of a lysis buffer containing 1% Nonidet P-40, 0.1% SDS, 50 mM Tris, pH 7.5, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 20 mM ß-glycerophosphate, 1.0 mM sodium orthovanadate, 10 µg/ml aprotinin, and 10 µg/ml leupeptin, for 20 min at 4°C. The protein concentration was determined using the Bio-Rad protein assay kit. An identical amount of protein (30 µg) from each lysate and culture supernatant was subjected to 10% SDS-PAGE.

The fractionated proteins were transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL, USA), and the filters were blocked for 1.5 h using nonfat dried milk in Tris-buffered saline (TBS: 50 mM Tris, 0.15 M NaCl, pH 7.5) containing 0.1% Tween 20, washed with TBS, and incubated at room temperature for 2 h at 1:50 dilution of rabbit anti-COX-2 monoclonal antibody (mAb), or 1:2000 dilution of rabbit anti-phospho-ERK1/2 and phospho-p38 MAPK mAb. The membranes were further incubated with a 1:2000 dilution of donkey anti-rabbit immunoglobulin G (IgG) antibody for 20 min, coupled with HRP. An enhanced chemiluminescence system (Amersham) was used for detection. Filters were subsequently exposed to film for 15 s, and the latter was processed.

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR) assay. Total cellular RNA was extracted from synovial fibroblasts using guanidinium thiocyanate and phenol (RNAzol B; Cinna/Biotek Labs Int., Friendswood, TX, USA). First-strand cDNA was synthesized by reverse transcription at 39°C for 50 min in a 20 µl reaction mixture containing 1 µl of total RNA, M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA), random primer (Takara Shuzo Co., Shiga, Japan), and RNase inhibitor (Toyobo Co.). Two microliters of denatured cDNA were amplified in a 20 µl final volume containing 1 U Taq DNA polymerase (Gibco BRL), 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 94°C for 1 min, 56°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 following specific primers were used for COX-2[15]: 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3'(forward), 5'-AGATCATCTCTGCCTGAGTATCTT-3'(reverse). Predicted size of the fragment was 301 bp. For ß-actin: 5'-CAAGAGATGGCCACGGCTGCT-3' (forward), 5'-TCCTTCTGCATCCTGTCGGCA-3'(reverse). Predicted size of the fragment was 275 bp.

NF-κB DNA binding activity. By ELISA, p65 DNA binding activity was measured with 10 µg of nuclear extract with the Trans-AM™ kit (Active Motif, Rixensart, Belgium) using the protocol supplied by the manufacturer and as described[16].

RESULTS

To examine the effect of EM on COX-2 expression in synovial tissue, second-passage synovial cells were cultured with (Figure 1, lanes 2–6) or without EM for 24 h, and lysates were analyzed by anti-COX-2 immunoblot. As shown in Figure 1, RA synovial cells constitutively expressed COX-2 (lane 1), and EM (0.1–1000 nM) suppressed COX-2 expression in a dose-dependent manner (lanes 2–6).

We also examined the effect of EM on cytokine-stimulated synovial cells. Synovial cells were stimulated by IL-1ß in the presence or absence of EM. Lysates were analyzed by anti-COX-2 immunoblot. Four-passage synovial cells did not express COX-2 constitutively (Figure 2, lane 1), while synovial cells stimulated by IL-1ß (0.1 ng/ml) expressed COX-2 (lanes 2–7). EM (0.1–1000 nM) suppressed COX-2 expression in a dose-dependent manner (lanes 3–7).

RT-PCR was performed to determine whether IL-1ß treatment induces COX-2 mRNA expression in synovial cells. Reverse transcription was performed on total RNA from synovial cells stimulated with IL-1ß for 6 h. COX-2 and ß-actin cDNA were amplified by PCR. There was a linear correlation between the number of cycles (n = 30) and

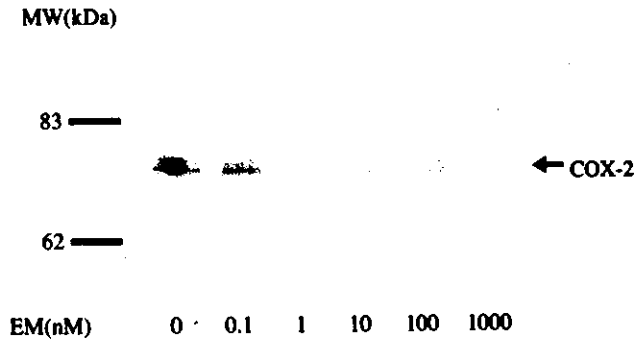

Figure 1. Effect of erythromycin (EM) on COX-2 expression of first-passage synovial cells with nonadherent cells. These cells were cultured in serum-free medium in the presence or absence of EM for 24 h and were not stimulated. Equal amounts (30 µg) of cell lysates were electrophoresed on 8% polyacrylamide gel and analyzed by Western blot using anti-COX-2 antibody. A representative example of 3 independent experiments is shown.

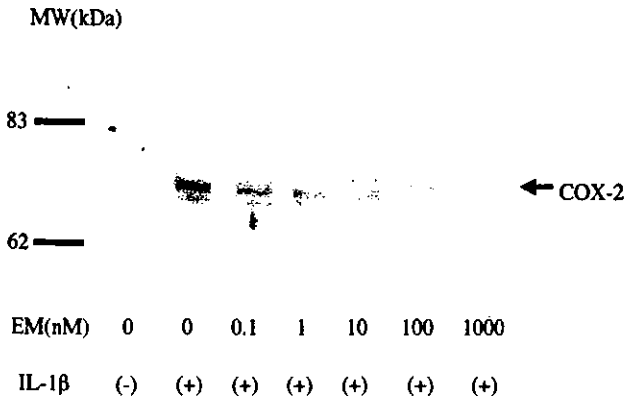

Figure 2. Effect of erythromycin (EM) on COX-2 expression of third-passage synovial cells. Cells were cultured in serum-free medium in the presence or absence of EM for 1 h. After treatment the cells were stimulated with IL-1ß for 24 h. Equal amounts (30 µg) of cell lysates were electrophoresed on 8% polyacrylamide gel and analyzed by Western blot using anti-COX-2 antibody. A representative example of 5 independent experiments is shown.

the yield of PCR products for both COX-2 and ß-actin mRNA. Stimulation of synovial cells by IL-1ß induced COX-2 mRNA expression in synovial cells (Figure 3, lane 2). EM pretreatment suppressed COX-2 mRNA induction by IL-1ß in synovial cells.

To investigate the role of NF-κB on COX-2 induction by IL-1ß in synovial cells, we performed p65 NF-κB DNA binding activity measured in an ELISA format using the Trans-AM™ kit. After synovial cells were cultured by serum-free RPMI with or without EM for 24 h, cells were stimulated by IL-1ß for 1 h. We observed that NF-κB reached the highest value rapidly over 1 h, and then it fell gradually over several hours. There was a significant difference between the group stimulated by IL-1ß and the group which was not stimuated by IL-1ß in the absence of EM ($p < 0.001$). EM did not affect the expression of NF-κB significantly (data not shown). IL-1ß-stimulated synovial cells showed enhanced DNA binding activity of NF-κB, and EM had no effect on such activity. We preincubated cells with or without EM for 2 h, and observed NF-κB activation. The result was the same as after 24 h incubation.

To determine whether the MAPK cascade is involved in the effects of EM on IL-1ß-modulated COX-2 induction, Western blotting was performed using anti-phospho-ERK1/2 antibody. After culture of synovial cells with or without EM for 24 h, cells were stimulated by IL-1ß for 10 min. IL-1ß treatment activated ERK1/2 phosphorylation (Figure 4, lane 2), which was not observed in untreated control synovial cells (Figure 4, lane 1). EM (0.1–1000 nM) did not affect IL-1ß-induced ERK1/2 phosphorylation in rheumatoid synovial cells (Figure 4, lanes 3–7).

We also examined the p38 MAPK cascade of IL-1ß-stimulated synovial cells. After pretreatment of synovial cells with or without EM for 24 h, cells were stimulated by IL-1ß for 10 min. IL-1ß treatment induced p38 MAPK phosphorylation (Figure 5, lane 2),. which was not detectable in untreated control synovial cells (Figure 5, lane 1). EM (0.1–1000 nM) inhibited IL-1ß-induced p38 phosphorylation of rheumatoid synovial cells in a dose-dependent manner (Figure 5, lanes 3–7).

DISCUSSION

Our study showed that EM downregulates COX-2 expression by inhibiting the p38 MAPK cascade, but EM did not inhibit the NF-κB or ERK1/2 cascade on IL-1ß stimulated rheumatoid synovial cells.

We investigated the effect of EM on second-passage synovial cells, which closely resemble *in vivo* conditions of synovial tissues. These cells were minced and dissociated by collagenase. It is possible that these cells were stimulated through mechanical processing. Accordingly, we cultured

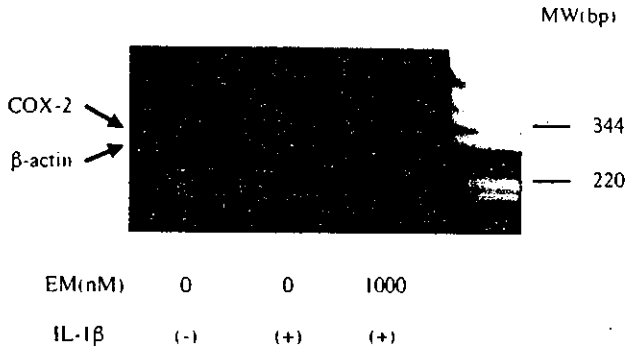

Figure 3. RT-PCR analysis for COX-2 mRNA of synovial cells treated with IL-1ß. Synovial cells were stimulated with IL-1ß (0.1 ng/ml) in the presence or absence of erythromycin (EM) for 6 h. Total RNA was reverse transcribed following PCR amplification for 30 cycles with specific primers for COX-2 and ß-actin. Representative example from 3 independent experiments is shown.

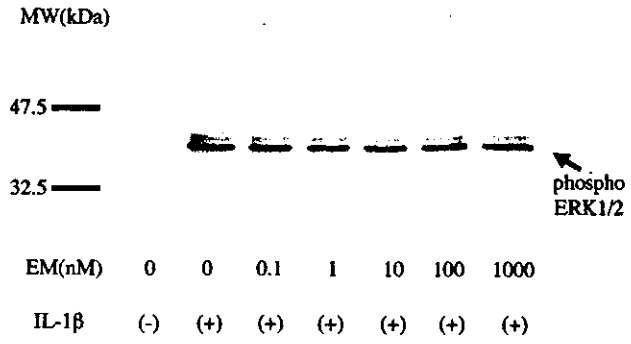

Figure 4. Effect of erythromycin (EM) on phospho-ERK1/2 expression of third-passage synovial cells. Cells were cultured in serum-free medium in the presence or absence of EM for 24 h. After treatment, cells were stimulated with IL-1ß for 60 min. Equal amounts (30 µg) of cell lysates were electrophoresed on 10% polyacrylamide gel and analyzed by Western blot using anti-phospho-ERK1/2 antibody. A representative example of 3 independent experiments is shown.

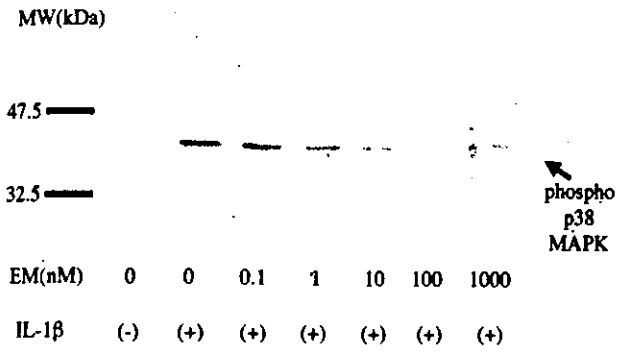

Figure 5. Effect of erythromycin (EM) on phospho-p38 MAPK expression of third-passage synovial cells. Cells were cultured in serum-free medium in the presence or absence of EM for 24 h. After treatment, cells were stimulated with IL-1ß for 60 min. Equal amounts (30 μg) of cell lysates were electrophoresed on 10% polyacrylamide gel and analyzed by Western blot using anti-phospho-p38 MAPK antibody. A representative example of 3 independent experiments is shown.

these cells for 24 h to reduce any effect of mechanical stimulation. The results revealed that freshly isolated synovial cells expressed COX-2 and that EM could suppress this COX-2 expression in synovial tissues.

Previous studies described that inflammatory cytokines stimulated prostaglandin production by inducing COX-2 expression in fibroblasts[17], macrophages[18], and chondrocytes[19], all cell types present in synovial tissues. We investigated whether EM could alter IL-1ß-mediated COX-2 expression on 4-passage synovial cells. The latter cells did not express COX-2. Therefore we stimulated synovial cells with IL-1ß to induce COX-2 expression, which was examined by Western blotting and RT-PCR. The results revealed that EM suppressed COX-2 protein expression, which may contribute to the PGE_2 synthesis in rheumatoid synovium.

Overexpression of COX-2 mRNA and protein observed in the RA synovium is thought to be etiologically related with the disease process[20]. COX-2, an inducible form of cyclooxygenase, is upregulated *in vitro* by various proinflammatory agents, such as IL-1ß and tumor necrosis factor-α. COX-2 appears to be responsible for the increase in prostaglandin synthesis at the site of inflammation[21]. Crofford, *et al*[20] reported that COX-2 was expressed in infiltrating mononuclear cells, endothelial cells of blood vessels, and subsynovial fibroblast-like cells. This effect of EM on COX-2 expression was reported in murine macrophages stimulated with lipopolysaccharide[22]. Additionally, we observed cell viability with or without EM at any state. In the quantity we used, EM did not affect cell viability (data not shown). Because we observed that EM had no effect for COX-1 expression (data not shown), this inhibitory effect of EM seems to be specific for COX-2. Our results suggest that EM can attenuate the inflammatory process in synovium.

Three pathways, NF-κB, ERK1/2, and the p38 MAPK cascade, are known to induce COX-2 expression in IL-1ß-stimulated synovial cells. We investigated the exact pathway that could contribute to EM suppression of COX-2 expression.

NF-κB is an important transcriptional factor for IL-1ß-induced COX-2 gene expression, and there is some evidence suggesting that NF-κB also mediates transcriptional induc-

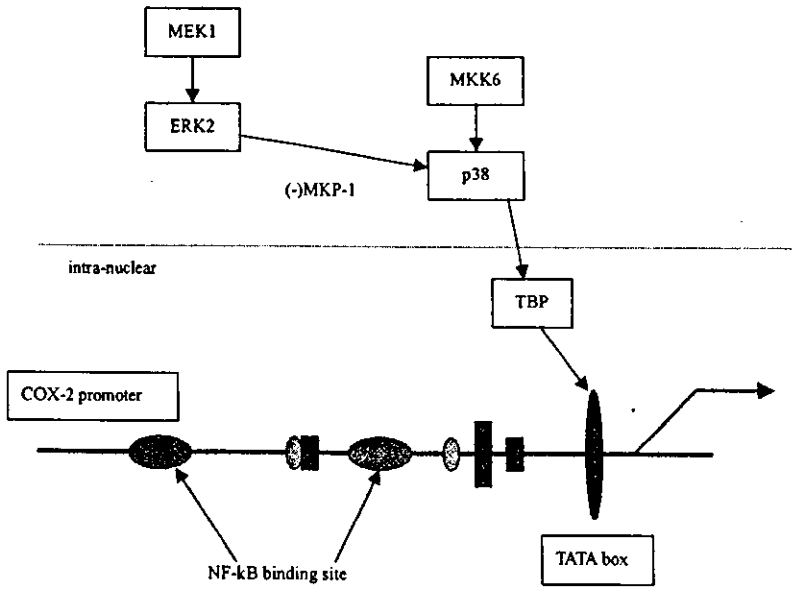

Figure 6. The interrelationships involved in COX-2 gene transcription. The p38 MAPK positively regulates and ERK kinase negatively regulates COX-2 expression through NF-κB-dependent gene transcription through TATA binding protein (TBP).

tion of the COX-2 gene in IL-1ß-stimulated human RA synovial fibroblasts[23]. EM inhibits transcriptional activation of NF-κB through calcineurin-independent signaling of phorbol-12-myristate 13-acetate (PMA) and calcium ionophore-stimulated T cells[24]. Our study, however, revealed that EM suppressed COX-2 protein expression in IL-1ß-stimulated synovial cells without affecting the DNA binding activity of NF-κB.

In smooth muscle cells, ERK are involved in early signal transduction pathways through which IL-1ß increases PGE_2 synthesis through the induction of COX-2[25]. The ERK and p38 MAP kinases have differential effects on NF-κB-driven transcription. The ERK pathway negatively regulates NF-κB-driven transcription, in part, by inhibiting p38 MAP kinase activity through MAPK phosphatase-1 expression[26]. In our study, EM did not alter the activation of ERK1/2 phosphorylation. These results suggest that EM can inhibit p38 MAPK phosphorylation without affecting the ERK pathway.

Previous studies showed that p38 MAPK mediates not only a transcriptional response, presumably at the level of the COX-2 promoter, but also stabilization of COX-2 mRNA[27,28]. Thus, the p38 MAPK cascade in IL-1ß-stimulated human synovial fibroblasts induces a positive feedback, such as PGE_2-dependent stabilization of COX-2 mRNA[29]. Ridley, *et al*[30] demonstrated in HeLa cells that a p38 MAPK inhibitor regulated the stability of IL-1-induced COX-2 mRNA. The COX-2 mRNA promoter has a NF-κB binding site and TATA box. The p38 MAPK positively regulates NF-κB-dependent gene expression by modulating the phosphorylation and subsequent activation of TATA binding protein (TBP)[31]. In our study, EM inhibited IL-1ß-induced p38 MAPK activation in rheumatoid synovial cells. Other investigators have reported that tetracycline[32] and dexamethasone[33] could also regulate controlled COX-2 mRNA by inhibition of the p38 MAPK pathway. In contrast, nonsteroidal antiinflammatory drugs (NSAID) directly inhibit the action of COX to produce prostaglandins[34,35]. In addition, some NSAID such as sodium salicylate and aspirin inhibited the activation of NF-κB[36].

It is well known clinically that longterm administration of low dose EM is very effective against diffuse panbronchiolitis (DPB)[37] and increases the survival rate of patients[38]. In addition, EM inhibits neutrophil chemotaxis in bronchioles and alveoli of DPB[39], and modulates IL-8 expression in normal and inflamed human bronchial epithelial cells[40]. Considering these data, EM might be effective in the treatment of RA through its antiinflammatory actions.

We demonstrated that erythromycin suppressed COX-2 expression in IL-1ß-stimulated synovial cells by inhibiting p38 MAPK phosphorylation. These results suggest that the antiinflammatory action of erythromycin could suppress joint inflammation in RA by a mechanism different from that of NSAID.

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The role of IL-18 in the modulation of matrix metalloproteinases and migration of human natural killer (NK) cells

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Abstract In this study, we examined whether interleukin-18 (IL-18) affects natural killer (NK) cells' migration and matrix metalloproteinases (MMPs) production. We demonstrated that chemotaxis of human NK cells through basement membrane-like Matrigel was augmented by IL-18. As well, IL-18 stimulation induces the production of activated forms of matrix metalloproteinase-2 (MMP-2) as well as the production of pro-MMP-2 from NK cells. We also demonstrated that MT1-MMP expression on human NK cells, which is a major activator of MMP-2, was induced by IL-18 stimulation coordinated with MMP-2 activation. These data suggest that the MT1-MMP/MMP-2 system participates in the degradation of basement membrane components and thus contributes to NK cell migration.

Keywords: Interleukin-18; Matrix metalloproteinase; Membrane-type matrix metalloproteinase; NK cell

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc endo-proteinases that are a secreted or membrane-bound form of protein [1,2]. They play important roles in many biological processes including angiogenesis, inflammation and cancer metastasis [3–5]. The main characteristics of MMPs is the degradation of the extracellular matrix of basement membranes, thus enabling cells to invade into tissues [6–8]. For example, T cells secrete the gelatinases, MMP-2 and MMP-9 in response to cytokines and inflammatory mediators and migrate across basement membrane by degrading extracellular matrix (ECM) using gelatinases [9–11].

Natural killer (NK) cells are endowed with the ability to express spontaneous cytotoxicity against tumor cells or virus-infected cells [12]. Upon several biological responses such as virus infection and carcinogenesis, NK cells encompass movements directed against target cells [13–15]. They should possess proteinases which mediate their transmigration through the ECM component for their migration to the target cells [16–18]. Recent studies have suggested that matrix metalloproteinases play an important role in basement membrane degradation and the transmigration of NK cells [19]. The 92 kDa gelatinase (gelatinase B, MMP-9) and the 72 kDa gelatinase (gelatinase A, MMP-2), which efficiently degrade native collagen type 4 and 5, are believed to play an important role in lymphocyte migration [20]. Recently, membrane-type MMP (MT-MMP) has been shown to be a potent activator of MMP-2 by cleaving the propeptide domain [21,22]. It has also been demonstrated that IL-2 activated NK cells produce MMP-2 and MMP-9 and express MT-MMP [23,24]. Therefore, IL-2 augments NK cells' migration as well as NK cells' cytotoxicity. IL-18 is also essential for NK cells' cytotoxicity and antitumor activity [25,26]. It is probable that IL-18 augments NK cells' migratory ability by producing MMPs in addition to its cytotoxicity. In order to identify the contribution of IL-18 on NK cells' migratory function, we examined the MMPs and MT-MMPs produced by IL-18-stimulated human NK cells.

2. Materials and methods

2.1. Reagents

Recombinant human IL-18 was purchased from MBL (Nagoya, Japan). Endotoxin levels were less than 0.1 ng per 1 mg recombinant IL-18 protein. Recombinant human IL-12 was purchased from Genzyme (Cambridge, MA). Recombinant human IL-2 was kindly provided from Shionogi Pharmaceutical Co. (Osaka, Japan). Anti-human IL-18 neutralizing monoclonal antibodies (125-2H) were purchased from MBL. Anti-human IL-2 neutralizing monoclonal antibodies (5334.21) were purchased from Genzyme. Anti-human IFN-γ neutralizing monoclonal antibodies (NIB42) were purchased from eBioscience (San Diego, USA).

2.2. Isolation of NK cells

NK cells were purified from buffy coat cells (kindly provided by the Nagasaki Red Cross Blood Center, Nagasaki, Japan) isolated from peripheral blood, as previously described [27]. Briefly, peripheral blood mononuclear cells (PBMC) were separated from whole blood by centrifugation over Ficoll–Hypaque. For NK cell isolation, cells were depleted of T lymphocytes, B lymphocytes, and macrophages/monocytes using a NK cell isolation kit (Minitenyi Biotec, Bergisch Galdbach, Germany), including bead-coupled mAb against CD3, CD4, CD19, and CD33. Using a MACS magnetic separator (Miltenyi Biotec), NK cells were purified phenotypically >95% $CD16^+$ $CD56^+$, as determined by flow cytometry. Freshly isolated NK cells were cultured with serum-free media (RPMI 1640), with or without IL-18, at a concentration of 1×10^6 cells/ml in 24-well plates (Costar, Cambrige, MA).

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Abbreviations: IL-18, interleukin-18; MMPs, matrix metalloproteinases; MT-MMP, membrane-type matrix metalloproteinase; NK cells, natural killer cells; TIMP, tissue inhibitor of matrix metalloproteinase

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2.3. Migration assay

Cells migration was quantified using Transwell inserts (6.5 μm; Costar, Cambridge, MA) fitted with polycarbonate filters (8-μm pre size; Nuclepore Corp., Pleasanton, CA). The upper sides of the filter were coated with Matrigel (200 μg/cm^2, Collaborative Research Inc, Bedford, MA) and dried overnight. NK cells (5×10^6 in 1000 μl medium) were added to the upper compartment of the insert with or without IL-18. The lower compartment contained 1500 μl of medium containing identical concentrations of IL-18. The chambers were incubated at 37 °C for 24 h. Then, the numbers of NK cells that had migrated into the lower chamber were counted.

2.4. Gelatin zymography

Culture media were incubated at 37 °C for 20 min in sodium dodecyl sulfate (SDS) sample buffer free of reducing agents and then electrophoresed on 8% polyacrylamide gels containing 0.5% gelatin at 4 °C. After electrophoresis, the gels were washed in 2.5% Triton-X 100 to remove SDS and incubated with 50 mM Tris–HCl buffer (pH 7.5) containing 0.15 M NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3 for 16 h at 37 °C, and then stained with 0.1% Coomassie Blue R250. The gelatinolytic bands were analyzed by densitometer (Photometrics, Tucson, Arizona). Images from densitometer were transferred to a personal computer and analyzed using IPLab Gel software (Signal Analytics Corporation, Vienna, VA).

2.5. Immunoblot analysis

The expression of MT-MMP on NK cells and the secretions of tissue inhibitor of MMPs (TIMPs) from NK cells were analyzed by immunoblot. For this purpose, cells were washed with cold PBS and lysed by the addition of a lysis buffer containing 1% Nonidet P-40, 50 mM Tris (pH 7.5), 100 mM NaCl, 5 mM EDTA, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 20 min at 4 °C. Insoluble material was removed by centrifugation at × 1500 *g* for 15 min at 4 °C. The supernatant was saved and the protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). An identical amount of protein (30 μg) from each lysate or culture supernatant was subjected to 10% SDS–polyacrylamide gel electrophoresis. The fractionated proteins were transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL), and the filters were blocked for 1.5 h using non-fat dried milk in Tris-buffered saline (TBS: 50 mM Tris, 0.15 M NaCl, pH 7.5) containing 0.1% Tween 20, washed with TBS, and incubated at room temperature for 2 h at a 1:150 dilution of mouse anti-MT1, 2, 3-MMP or TIMP-1, 2 monoclonal antibodies (Fuji Chemicals, Takaoka, Japan). The membranes were further incubated with a 1:2000 dilution of horseradish peroxidase-conjugated donkey anti-mouse immunoglobulin G (IgG) antibody (Promega, Madison, WI) for 20 min. An enhanced chemiluminescence (ECL) system (Amersham) was used for detection. The filters were subsequently exposed to film for 15 s and the latter was processed.

2.6. RNA preparation and RT-PCR assay

Total cellular RNA was extracted from NK cells 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 (Gibco-BRL, Gaithersburg, MD). 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 by a 20 μl final volume containing 1 U *Taq* DNA polymerase (Gibco-BRL), 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 following specific primers were used for MT1-MMP:
5′-AGGCGCCCCCGATGTGGTGTT-3′ (forward),
5′-TGGCCGAGGGGTCACTGGAATGCT-3′ (reverse).
Predicted size of the fragment is 502 bp (corresponding base pairs: 265–766).

For β-actin:
5′-GACGAGGCCCAGAGCAAGAGAG-3′ (forward),
5′-ACGTACATGGCTGGGGTGTTG-3′ (reverse).
Predicted size of the fragment is 284 bp.

3. Results

3.1. Migration of IL-18-stimulated human NK cells

We used a matrigel invasion assay to determine the ability of IL-18-stimulated human NK cells to invade through a model basement membrane. Freshly isolated human NK cells were placed in a Matrigel invasion chamber in the presence or absence of IL-18 for 24 h. As shown in Fig. 1A, IL-18 enhanced NK cell mobility significantly at respective concentrations of 0–100 ng/ml. IL-18 may have a capacity to induce IL-2, which has been shown to augment NK cell migration [11]. Therefore, we examined the effects of anti-IL-2 antibodies on IL-18-stimulated NK cell migration. As shown in Fig. 1B, anti-IL-2 antibodies did not affect IL-18-stimulated NK cell migratory activity. IL-12 is a proinflammatory cytokine produced by macrophages and augments NK cell cytotoxicity [28]. We investigated whether IL-12 induces NK cell migration. As shown in Fig. 1C, IL-12 induces NK cell migration and its migration-inducing activity is similar to those of IL-18. IL-12 has been shown to enhance IL-18-mediated biological activity by inducing IL-18R expression [29]. Therefore, we examined the combined effects of IL-12 and IL-18 on NK cell migration. The combined treatments of IL-18 plus IL-12 upregulates NK cell migratory activity (Fig. 1C).

3.2. Gelatin zymographic analysis of NK cell culture supernatants

We sought to determine whether or not IL-18 enhances the MMP secretion necessary to allow NK cells to migrate across the Matrigel mode basement membrane. Supernatants isolated from IL-18-stimulated NK cells grown in serum-free media were analyzed using SDS-PAGE gelatin zymography. The results indicated two major gelatin-cleaving activities that correspond to the 72-kDa MMP-2 and 92-kDa MMP-9. These two gelatinolytic bands were increased by IL-18 stimulation (Fig. 2A). Gelatinases are known to be secreted in a latent form and converted to the active form through the cleavage of the N-terminal pro-peptide domain by membrane-type matrix metalloproteinase [21,22]. The enzymatic ability of gelatinases is influenced by these activation processes. In gelatin zymography of IL-18-stimulated NK cell culture supernatants, a detectable gelatinolytic band was also noted at about 66 kDa in addition to the 72 kDa MMP-2. This lower product of 66 kDa corresponds to the active form of MMP-2. It is possible that IL-18 stimulates other cytokines, such as IL-2, production from NK cells. As shown in Fig. 2B, IL-2 stimulated the gelatinases secretion from NK cells. To determine whether NK cells secreted gelatinases in response to IL-18 or indirectly via IL-2, IL-2 was blocked by anti-IL-2 neutralizing antibodies. Although anti-IL-18 neutralizing antibodies blocked the IL-18-induced secretion of activated MMP-2, anti-IL-2 neutralizing antibodies did not affect this secretion from NK cells (Fig. 2B and C). IL-18 also induces IFN-γ production from NK cells [30]. To determine whether IL-18-mediated IFN-γ production affects the MMPs secretion from NK cells, we examined the effects of anti-IFN-antibodies on IL-18-induced activated MMP-2 secretion. As shown in Fig. 2D, anti-IFN-γ neutralizing antibodies did not affect IL-18-induced activated MMP-2 secretion from NK cell.

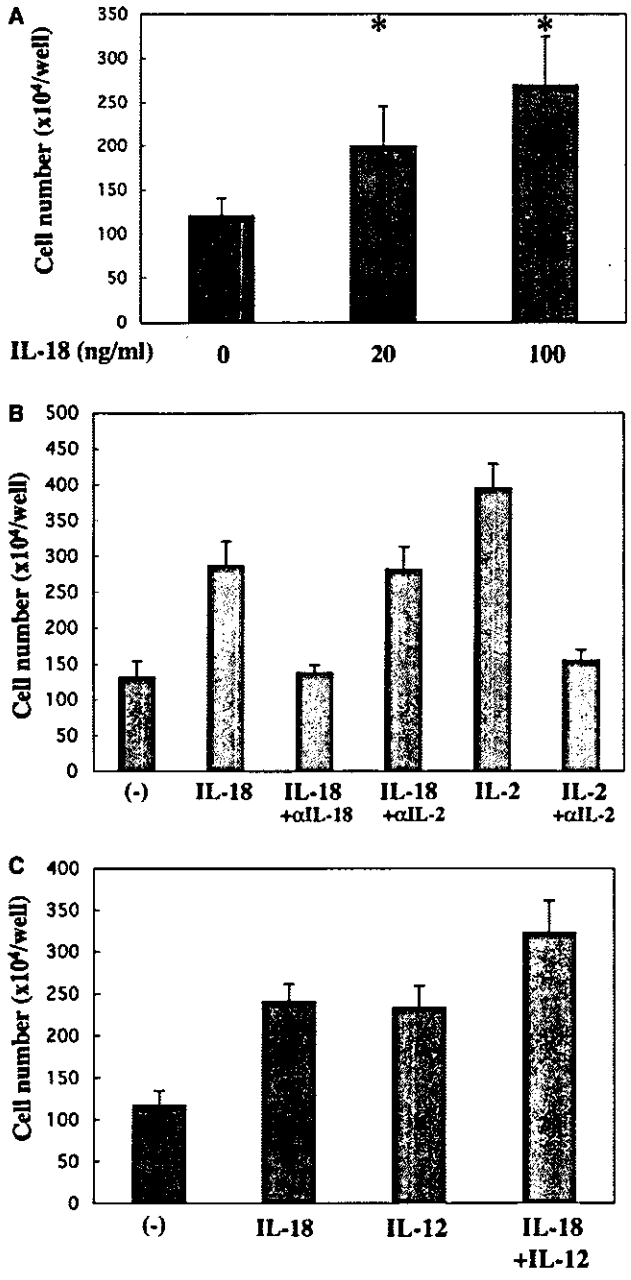

Fig. 1. (A) Effects of IL-18 on human NK cell migration across a model of basement membrane. Human NK cells (5×10^6) in 1.0 ml were placed in the top well of Matrigel invasion chambers with various concentrations of IL-18 for 24 h. Results are expressed as cell numbers invaded into bottom chambers through Matrigels. Data for bar graphs represent means ± S.E.M. of three independent experiments. Statistical significance was performed by Student's *t*-test. $^*P < 0.05$ versus control (untreated NK cells). (B) Effects of anti-cytokine antibodies on NK cell migration across a model of basement membrane. Human NK cells (5×10^6) in 1.0 ml were placed in the top well of Matrigel invasion chambers with IL-18 (100 ng/ml) in the presence or absence of anti-IL-2 (αIL-2 final concentrations; 2 μg/ml) or anti-IL-18 (αIL-18 final concentrations; 10 μg/ml) neutralizing antibodies for 24 h. Results are expressed as cell numbers invaded into bottom chambers through Matrigels. Data for bar graphs represent means ± S.E.M. of two independent experiments. (C) Combined effects of IL-12 and IL-18 on NK cell migration across a model of basement membrane. Human NK cells (5×10^6) in 1.0 ml were placed in the top well of Matrigel invasion chambers with IL-18 (100 ng/ml) and IL-12 (10 ng/ml) for 24 h. Results are expressed as cell numbers invaded into bottom chambers through Matrigels. Data for bar graphs represent means ± S.E.M. of two independent experiments.

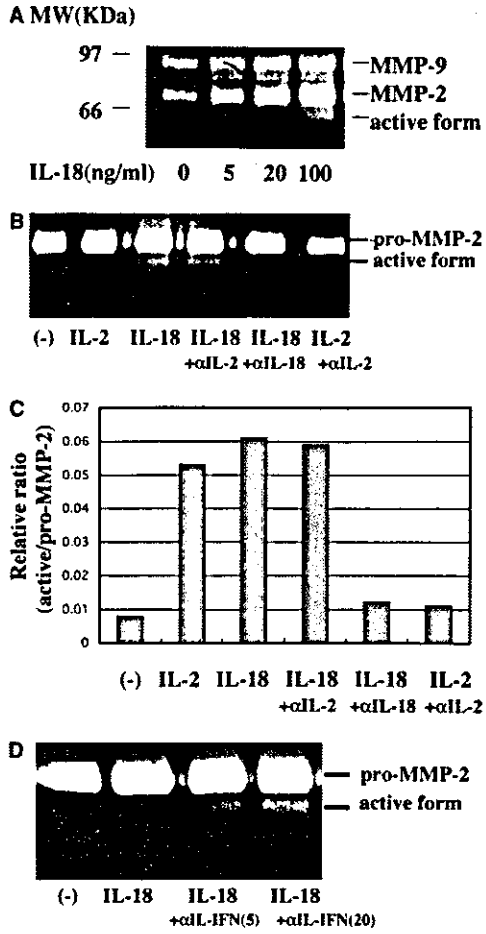

Fig. 2. (A) IL-18-induced gelatinase secretion from human NK cells. Human NK cells were cultured with IL-18 in serum-free culture media for 24 h. Conditioned media were analyzed by gelatin zymography. Note the activated MMP-2 with lower molecular weights in addition to 72 kDa MMP-2 in IL-18-stimulated NK cells-conditioned media. The data shown are representatives of three independent experiments. (B,C) Effects of anti-cytokine antibodies on gelatinases secretion from NK cells. (B) Human NK cells were cultured with IL-18 (100 ng/ml) or IL-2 (100 IU/ml) in the presence or absence of anti-IL-2 (αIL-2) or anti-IL-18 (αIL-18) neutralizing antibodies for 24 h. Conditioned media were analyzed by gelatin zymography. Note the activated MMP-2 with lower molecular weights in addition to 72 kDa MMP-2 in IL-18-stimulated NK cells-conditioned media. The data shown are representatives of two independent experiments. (C) Densitometric analyses of B are presented as the relative ratio of active form/pro-form of MMP-2. (D) Effects of anti-IFN-γ antibodies on gelatinases secretion from NK cells. Human NK cells were cultured with IL-18 (100 ng/ml) in the presence or absence of anti-IFN-γ neutralizing antibodies (final concentrations; 2 μg/ml, 10 μg/ml) for 24 h. Conditioned media were analyzed by gelatin zymography. Note the activated MMP-2 with lower molecular weights in addition to 72 kDa MMP-2 in IL-18-stimulated NK cells-conditioned media. The data shown are representatives of two independent experiments.

We analyzed the presence of other MMPs in NK-cell conditioned media. However, we could not detect MMP-3 and MMP-13 in NK cell conditioned media with or without IL-18 by immunoblot analysis (data not shown).

3.3. Expression of MT-MMP

MT-MMP, which is expressed on the cell surface, has the ability to convert the latent form of MMP-2 to the active form

[21]. In order to confirm the expression of MT1-MMP on NK cells, we performed immunoblot analysis using a specific antibody. Cellular lysates prepared from IL-18-stimulated or unstimulated NK cells were analyzed by immunoblot using anti-MT1-MMP monoclonal antibodies. As shown in Fig. 3A, the expression of MT1-MMP was faintly detected in unstimulated NK cells. As shown in Fig. 3A, IL-18 stimulation induced the expression of MT1-MMP in NK cells in a dose-dependent manner. This IL-18 induced MT1-MMP expression on NK cells was completely inhibited by anti-IL-18 antibodies (Fig. 3B). Although MT2-MMP was also detected in NK cells, its expression was not affected by IL-18 stimulation (Fig. 3C). MT3-MMP was not detected in unstimulated or IL-18-stimulated NK cells (data not shown). We also tried to determine whether IL-18 treatment induces MT1-MMP mRNA expression in human NK cells using RT-PCR analysis. MT1-MMP and β-actin cDNA were amplified by PCR. MT1-MMP mRNA expression was induced in IL-18-treated NK cells but not in untreated NK cells. No change was noted in the amplified fragments of the housekeeping gene β-actin (Fig. 4).

3.4. TIMPs secretion from IL-18-treated NK cells

The enzymatic activities of gelatinases are also influenced by a family of TIMPs. Therefore, we analyzed the secretion of TIMPs from IL-18-stimulated NK cells using immunoblot analysis. Although TIMP-1 and TIMP-2 proteins were identified in NK cells-conditoned media, these secretions were not altered with or without IL-18 stimulation (data not shown).

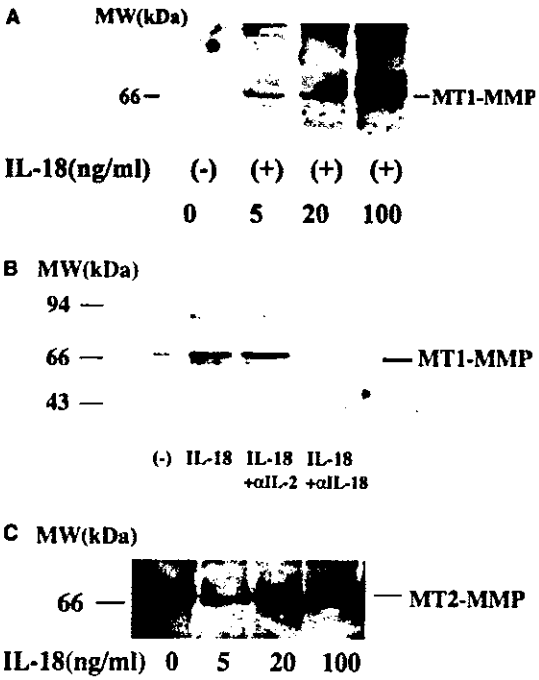

Fig. 3. (A,B) Immunoblot analysis of MT1-MMP in IL-18-treated human NK cells. Human NK cells were cultured with IL-18 in the presence (B) or absence (A) of anti-IL-18 neutralizing antibodies for 24 h. Blots of SDS–PAGE gels of IL-18-treated NK cell lysates were probed with antibodies to human MT1-MMP. The data shown are representatives of three independent experiments. (C) Immunoblot analysis of MT2-MMP in IL-18-treated human NK cells. Human NK cells were cultured with IL-18 for 24 h. Blots of SDS–PAGE gels of IL-18-treated NK cell lysates were probed with antibodies to human MT2-MMP. The data shown are representatives of two independent experiments.

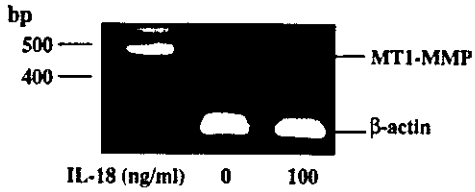

Fig. 4. RT-PCR analysis of mRNA for MT1-MMP in NK cells. Human NK cells were treated with or without IL-18 (100 ng/ml) for 12 h. Total RNA was reverse transcribed following PCR amplification with primer for MT1-MMP and β-actin. Ethidium bromide staining of PCR products. The data shown are representatives of three independent experiments.

4. Discussion

IL-18, originally designated as IFN-γ inducing factor, is a cytokine produced by activated macrophages, monocytes, and dendritic cells during innate immune response [31,32]. IL-18 has been shown to have the capacity to amplify IFN-γ secretion by NK and NKT cells [25,26]. Although NK cell numbers are normal in IL-18$^{-/-}$ mice, NK cell function including cytotoxicity has been shown to be significantly reduced [33]. Collectively, these data demonstrated that IL-18 is essential for NK cell function. We sought to determine whether IL-18 enhances NK cell cytotoxicity by inducing transmigration or interaction with ECM. IL-18 stimulation significantly augments the migration of human NK cells through a layer of Matrigel by degrading the constituents of the Matrigel.

The migration of immune cells including T cells has been previously shown to be mediated by MMPs [34]. MMPs are members of a family of Zn^{2+}-dependent endopeptidases and exhibit full activity for a wide range of connective matrix proteins [2]. In gelatin zymography, the active form of MMP-2 as well as pro-MMP-2 was detected in IL-18-stimulated NK cell culture media. MMPs activity is induced by a coordinated increase in transcription, secretion and proteolytic activation [35]. During the proteolytic process, latent pro-MMPs are activated by cleavage of the pro-peptide domain and conformational change of the Zn^{2+}-binding motif [36]. Various factors including serine proteinases are known to activate pro-MMP. However, pro-MMP-2 is unique in that it is not activated by serine proteinases, but is activated by MT-MMP [22]. MT1-MMP has been identified as a putative cell membrane-associated activator of pro-MMP-2 and shown to be expressed in IL-2-activated NK cells [24].

One of the interesting findings of the present study is that pro-MMP-2 is secreted as an active form in IL-18-stimulated NK cells, suggesting that pro-MMP-2 is activated on NK cells. Murine IL-2-activated NK cells was shown to express MT1-MMP by immunoblot analysis [24]. Therefore, we investigated the expression of MT-MMP in IL-18-stimulated NK cells. Our data clearly indicated that IL-18 induced MT1-MMP expression on human NK cells. These data suggest that IL-18-stimulated NK cells not only secrete pro-MMP-2 but also efficiently convert pro-MMP-2 to an active form by expressing

MT1-MMP on its surface. Because MT1-MMP, MT2-MMP, and MT3-MMP are believed to be tissue activators of pro-MMP-2 [37], we examined their protein expression on NK cells by immunoblot analysis. Although MT1-MMP is induced in IL-18-stimulated NK cells, MT2-MMP, which is constitutively expressed in NK cells, was not increased by IL-18. The activation activity of MT3-MMP is much weaker than that of MT1-MMP [38]. The increase in MT1-MMP protein expression in NK cells was much larger than that expected from mRNA. Upregulation of MT1-MMP protein level in IL-18-treated NK cells occurred at least at the pre-translational level as shown by the increased MT1-MMP mRNA. However, additional post-translational regulation cannot be ruled out, since post-translational upregulation mechanism of MT1-MMP protein has been described [39].

This is the first report demonstrating that IL-18 clearly induced MMP-2 secretion and MT1-MMP expression using freshly isolated human NK cells. These data suggest that both gelatinases and MT1-MMP might be biologically important in the penetration of NK cells through the basal lamina. In summary, we showed that IL-18 potentiated NK cells' migratory ability by inducing MMP-2 production but also by promoting MMP-2 activation, probably by inducing MT1-MMP expression. This IL-18-mediated modulation of MMP-2 and MT1-MMP suggests that this cytokine may play a potential role in NK cell migration.

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

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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-

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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-

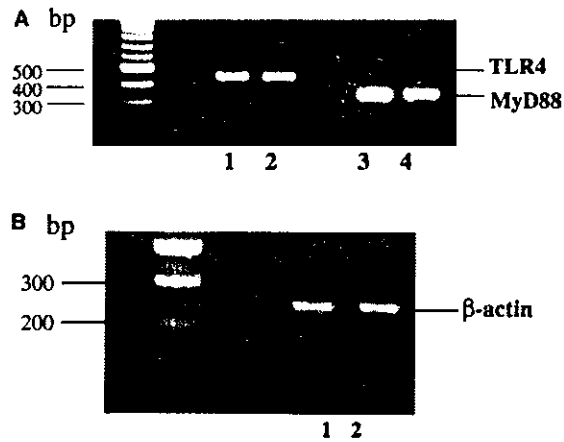

Fig. 1. Expression of TLR4 and *MyD88* mRNA in human hepatocytes. (A) Total RNA was obtained from THP-1 cells (lanes 1 and 3) and human hepatocytes and TLR4 and *MyD88* mRNA were analyzed by PCR following reverse transcription. (B) β-Actin expression in THP-1 cells (lane 1) and human hepatocytes (lane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

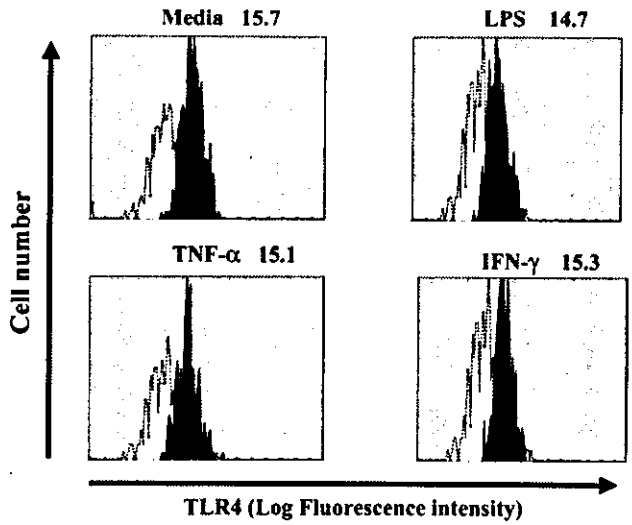

Fig. 2. Effects of cytokines on TLR4 expression on human hepatocytes. Human hepatocytes were treated with media, LPS (100 ng/ml), TNF-α (100 ng/ml) or IFN-γ(100 ng/ml) for 24 h. Expression of TLR4 on hepatocytes was measured by flowcytometer and represented as changes in fluorescence between TLR4 and 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).

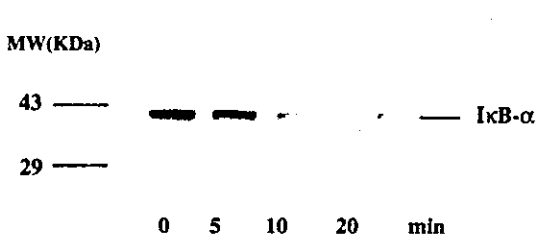

Fig. 3. LPS stimulated IκB-α degradation in hepatocytes. Human hepatocytes were stimulated with LPS (100 ng/ml) for the indicated times. Cells were lysed and cellular lysates were assessed by anti-IκB-α immunoblot analysis. The data shown are representatives of at least three independent experiments.

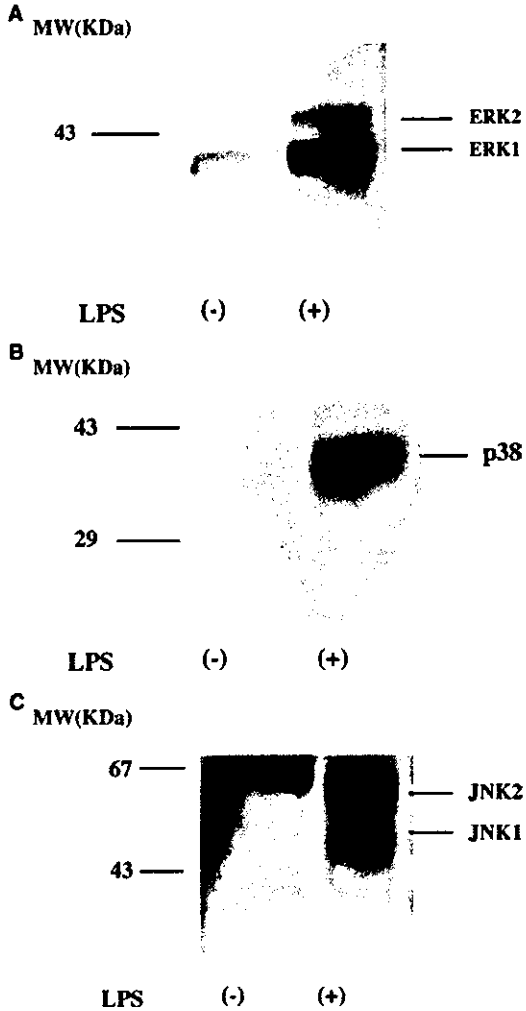

Fig. 4. LPS stimulation induces MAPK activation in hepatocytes. Quiescent human hepatocytes were stimulated with LPS (100 ng/ml) for 15 min. Cells were lysed and cellular lysates were analyzed by immunoblot using anti-phospho-specific ERK1/2 (A), p38 (B) and JNK1/2 (C) antibodies. The data shown are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

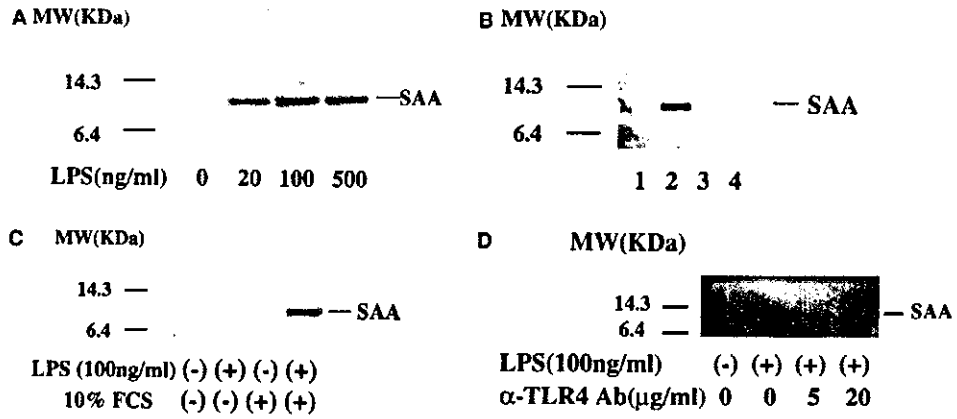

Fig. 5. (A) LPS induces SAA synthesis from human hepatocytes. Human hepatocytes were stimulated with the indicated concentrations of LPS for 24 h. SAA production was measured by anti-SAA immunoblot using culture supernatants. The data shown are representatives of at least three independent experiments. (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.

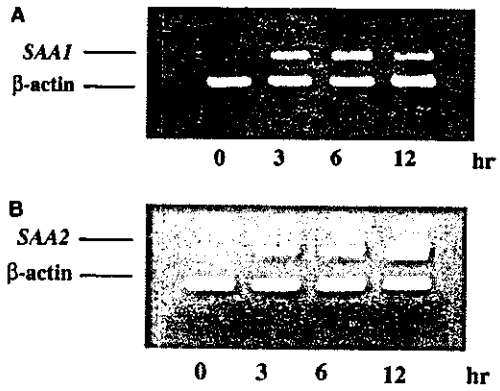

Fig. 6. LPS induces *SAA1,2* mRNA expression in human hepatocytes. Human hepatocytes were stimulated with LPS (100 ng/ml) for the indicated times. Expression on *SAA1* (A) and *SAA 2* (B) was analyzed by PCR following reverse transcription. β-Actin expression was used as control. The data shown are representatives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