Activation of Synovial Cell p38 MAP Kinase by Macrophage Migration Inhibitory Factor

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ABSTRACT. ***Objective.*** Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine important in animal models of rheumatoid arthritis (RA). We investigated the utilization by MIF of mitogen activated protein (MAP) kinase signalling pathways in the stimulation of fibroblast-like synoviocytes (FLS), cyclooxygenase-2 (COX-2), prostaglandin E_2 (PGE_2), and interleukin 6 (IL-6) and IL-8 expression.

Methods. Cultured human RA FLS were treated with recombinant MIF. Activation of MAPK was measured by Western blotting and blocked using specific inhibitors. The expression of COX-2, PGE_2, IL-6, and IL-8 were measured using flow cytometry, ELISA, and real-time polymerase chain reaction.

Results. MIF induced the phosphorylation of FLS p38 and extracellular-signal regulated kinase (ERK) MAP kinase. MIF significantly induced COX-2 and IL-6 protein and mRNA expression as well as PGE_2 and IL-8 production. Antagonism of p38 MAP kinase inhibited MIF induction of COX-2, PGE_2, and IL-6. In contrast, antagonism of ERK had no effect on COX-2, PGE_2, or IL-6. Neither antagonist inhibited MIF-induced IL-8.

Conclusion. MIF activates RA FLS COX-2 and IL-6 expression via p38 MAP kinase activation and induces IL-8 via p38 and ERK MAP kinase-independent pathways. (J Rheumatol 2004;31:1038–43)

Key Indexing Terms:
MIGRATION INHIBITORY FACTOR p38 MITOGEN ACTIVATED PROTEIN KINASE
CYCLOOXYGENASE-2 PROSTAGLANDIN E_2 INTERLEUKIN 6 INTERLEUKIN 8

Rheumatoid arthritis (RA) is a common, severe, chronic inflammatory disease. The inflamed joint is characterized by chronic inflammation and synovial hyperplasia. The initiating cause of RA is unknown, but it is clear that proinflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor (TNF) play important roles in RA pathology. Although specific anticytokine treatments directed against TNF and IL-1 are now available, the incomplete efficacy of these agents suggests that there remains much to learn about proinflammatory cytokines in RA.

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine released by a number of different cell types relevant to RA, including macrophages, T cells, and endothelial cells, and which is essential for both innate and adaptive immune responses[1,2]. We have previously reported the expression of MIF in human rheumatoid synovium, serum, synovial fluid, and cultured fibroblast-like synoviocytes (FLS)[3]. Moreover, MIF antagonism *in vivo* profoundly inhibits rat and mouse models of RA[4,5], and *in vitro* inhibits synoviocyte induction of monocyte TNF-α[3]. Together, these data suggest a potentially important role for MIF in the pathogenesis of RA[6].

The range of activities of MIF in RA and its cellular mechanism of action remain incompletely understood. We recently reported that MIF upregulates RA FLS expression of 2 genes involved in prostaglandin E_2 (PGE_2) synthesis, cytoplasmic phospholipase A_2 ($cPLA_2$) and cyclooxygenase-2 (COX-2), and that it has a role in IL-1-induced RA FLS activation[7]. Cytokines activate synoviocytes via signal transduction cascades including nuclear factor-κB (NF-κB) and MAP kinase pathways such as the extracellular-signal regulated kinase (ERK), p38, and SAPK/JNK pathways[8]. We reported that stimulation of RA FLS by MIF does not induce activation of the NF-κB signal transduction pathway[9]. Rather, at least as far as the phenomenon of FLS proliferation was concerned, MIF activation of FLS depended on the activation of the ERK MAP kinase pathway. The utilization of both NF-κB and MAP kinase-dependent pathways has been demonstrated in the stimulation of proinflammatory gene expression by other cytokines[10,11], and p38 MAP kinase in particular has been the subject of intense study in models of RA[12,13]. The production of COX-2 and PGE_2 by other cell types has been

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reported to be dependent on p38 phosphorylation[14,15]. Indeed, a major mechanism of action of p38 is the stabilization of COX-2 and other enzymes[11]. Significantly, p38 MAP kinase may also be involved in the production of proinflammatory cytokines implicated in RA, IL-6 and IL-8[16-20].

Involvement of the p38 pathway in diverse aspects of RA pathogenesis including cellular activation and inflammatory mediator and cytokine production prompted us to investigate the utilization of this pathway by MIF. No previous study has examined whether MIF activates p38 MAP kinase. Our study was thus designed to test the hypothesis that MIF activates RA FLS p38 MAP kinase, and to further define the utility of MAP kinase pathways in its activation of RA FLS. Given the recent interest in IL-6 as a therapeutic target in RA[16,18] and the known contributions of IL-8 in RA pathogenesis[17,19,20], we also undertook an examination of whether MIF could regulate FLS expression of these cytokines.

MATERIALS AND METHODS

Isolation and culture of FLS. FLS were obtained from synovium of patients with RA undergoing joint replacement surgery. Cultured FLS were isolated from synovial lining tissue as described[3]. In brief, a single cell suspension was obtained by enzyme digestion of minced synovial tissue using 2.4 mg/ml Dispase (grade II, 5 U/mg; Boehringer Mannheim, Melbourne, Australia), 1 mg/ml collagenase (type II; Sigma, Melbourne, Australia) and DNase (type I; Boehringer Mannheim). FLS were cultured in 10 cm culture plates in RPMI (ICN Biomedicals, Aurora, OH, USA)/10% fetal calf serum (FCS; Trace Biosciences Pty Ltd., Melbourne, Australia) at 37°C in a 5% CO_2 humidified incubator. Cells at third passage were more than 99% CD45–. Cells were used between passages 4 and 9. Unless otherwise indicated, FLS were seeded at the required concentration in RPMI/10% FCS and allowed to adhere overnight, prior to medium being replaced with RPMI/0.1% bovine serum albumin (BSA; Sigma) or unsupplemented RPMI for experimental purposes.

Analysis of MAP kinase activation. The phosphorylation of MAP kinases was assessed using Western blotting with monoclonal antibody (mAb) specific for the phosphorylated (activated) form of ERK and p38 MAP kinases, as described[9]. Cells were lysed in stress lysis buffer (20 mM HEPES, pH 7.7, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 20 mM ß-glycerophosphate, 100 mM NaCl, Triton X-100, 1 M DL-dithiothreitol (DTT), 0.1 nM sodium orthovanadate, 20 µg/ml leupeptin, 100 µg/ml phenylmethylsulfonyl fluoride, 20 µg/ml aprotinin; all reagents from Sigma) and disrupted by repeated aspiration through a 21-gauge needle. After 10 min incubation on ice, cells were centrifuged at 13,000 rpm for 15 min (4°C) and the supernatant/lysate was collected. Equal amounts of cellular proteins were boiled for 5 min in sodium dodecyl sulfate (SDS) loading buffer, fractionated on 10% SDS-polyacrylamide electrophoresis gels (Tris-glycine iGel; Monarch Medical, Queensland, Australia), and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Immunoblotting was performed using antibodies directed against the total and phosphorylated forms of ERK and p38 according to the manufacturer's instructions (Cell Signaling Laboratories, Beverly, MA, USA). Blots were then exposed to high performance chemiluminescence film (Hyperfilm ECL, Amersham, Arlington Heights, IL, USA) for 5 s to 20 min for detection.

Cell culture treatments. FLS were pretreated with an inhibitor of p38 MAP kinase SB203580 (5 µM)[13], an inhibitor of ERK phosphorylation PD98059 (50 µM)[21], or DMSO (vehicle control) for 30 min prior to stimulation with human recombinant MIF (100 ng/ml) (kindly donated by Prof. R. Bucala, Yale Medical School, New Haven, CT, USA). IL-1ß (0.1 ng/ml; Sigma) served as a positive control. After stimulation, cell culture supernatant, cell lysates, and cells were collected for protein and mRNA analysis.

Measurement of COX-2 protein. COX-2 protein expression was measured by permeabilization flow cytometry. Briefly, 2×10^5 cells were treated with MIF +/– MAPK inhibitors for 8 h. Cells were detached using Trypsin-EDTA (Sigma) then fixed in 2% paraformaldehyde (Sigma). Cells were permeabilized in 0.2% saponin (Sigma)/PBS then incubated sequentially with anti-COX-2 mAb (Cayman Chemicals, Ann Arbor, MI, USA) or isotype control (Dako, Glostrup, Denmark) and FITC-conjugated sheep anti-mouse immunoglobulin (Silenus, Melbourne, Australia). The labelled cells were analyzed on a Mo-flo cytometer (Cytomation, Fort Collins, CO, USA), and at least 5000 events were analyzed for each experiment. Results are expressed as the percentage of cells exhibiting fluorescence greater than a 5% cutoff set with negative control IgG-labelled cells (percentage positive), or the mean fluorescence intensity (MFI) of the total cell population after subtracting the MFI of control IgG-labelled cells.

Measurement of PGE_2 protein. PGE_2 protein was measured in cell culture supernatants by competitive ELISA (Neogen, Lansing, MI, USA). Positive signals were read at 450 nm against a standard curve for PGE_2 in the concentration range of 0.1 to 10.0 ng/ml. Duplicate cultures were used for each determination and results expressed in ng/ml PGE_2.

Measurement of IL-6 and IL-8. IL-6 and IL-8 proteins were measured in cell culture supernatant from 8 h cultures by sandwich ELISA using paired antibodies (R&D Systems, Minneapolis, MN, USA). Positive signals were read at 450 nm against a standard curve for each cytokine (R&D) in the concentration range of 7.8 to 500.0 pg/ml. Duplicate cultures were used for each determination and results expressed in pg/ml.

Measurement of COX-2 and IL-6 mRNA. Total RNA was extracted from cultured FLS using TRIzol (Gibco BRL, Grand Island, NY, USA); 0.5–1.0 µg of each RNA/sample were reverse-transcribed using Superscript II reverse transcriptase (Gibco BRL) and oligo dT18[7]. COX-2 and IL-6 mRNA expression was analyzed on a Roche LightCycler (Roche) using SYBR Green I as a double-stranded DNA-specific binding dye and continuous fluorescence monitoring as described[22]. In our study, human COX-2 and IL-6 dsDNA preparations from the extraction of polymerase chain reaction (PCR) products for human COX-2, IL-6, and ß-actin using the Qiaex II gel extraction system (Qiagen, Valencia, CA, USA) were employed as the assay standards. The level of expression of each mRNA and their estimated crossing points in each sample were determined relative to the standard preparation using the LightCycler computer software. A ratio of specific mRNA/ß-actin amplification was then calculated. Reagents for LightCycler PCR were purchased from Roche Biochemicals (Indianapolis, IN, USA). For PCR, 2 µl of each of the standard cDNA (diluted concentration 150, 15, 1.5, 0.15, 0.015 fg/ml) and the sample cDNA diluted 1:20 in sterile water were added to individual capillaries. Amplification was carried out in a total volume of 10 µl/each capillary containing primer concentrations of 3 pmol (COX-2, IL-6, and ß-actin) and 1 µl of NTPs, Taq enzyme, reaction buffer, and SYBR Green I dye, supplied in the LightCycler DNA Master SYBR Green I kit. The primer-specific nucleotide sequences and PCR reaction conditions were as follows: COX-2: F 5'-TCCTCCTGTGCCTGATGATT-3', R 5'-AACTGATGCGTGAAGTGCTG-3', annealing temperature 60°C; IL-6: F 5'-CTTCGGTCCAGTTGCCTTCT-3', R 5'-AGTGCCTCTTTGCTGCTTTC-3', annealing temperature 65°C; ß-actin: F 5'-AAACTGGAACGGTGAAGGTG-3', R 5'-AAACTGGAACGGTGAAGGTG-3', annealing temperature 60°C. Forty cycles of PCR were programmed. Melting curve analysis of amplification products was performed at the end of each PCR reaction. Control reactions for product identification consisted of analyzing the melting peaks (°C) in comparison to external standards for COX-2, IL-6, and ß-actin, and determining the length of the PCR products (bp) by agarose gel electrophoresis. Results are expressed as the ratio of COX-2 mRNA:ß-actin and IL-6 mRNA:ß-actin.

Statistical analysis. Results are expressed as the mean ± SEM. Statistical

analysis was performed using the Student t test, with values of $p < 0.05$ regarded as statistically significant.

RESULTS

Unstimulated RA FLS exhibited no constitutive phosphorylation of p38 MAP kinase (Figure 1A). Treatment of RA FLS with MIF induced the phosphorylation of p38 MAP kinase within 5 min, and this persisted until the latest timepoint studied, 120 min (Figure 1A). A slight reduction in p38 phosphorylation was consistently observed 30 min after MIF stimulation (Figure 1A). In contrast to the undetectable constitutive phosphorylation of p38 MAP kinase, constitutive phosphorylation of ERK MAP kinase in RA FLS was detected. Phosphorylation of ERK MAP kinase was further induced by MIF (Figure 1B).

We next examined the role of these MAP kinases in the regulation of RA FLS COX-2 expression. MIF significantly induced intracellular COX-2 protein content in cultured RA FLS ($p < 0.05$) (Figure 2A, 2B). The vehicle for MAP kinase inhibitors, DMSO, had no significant effect on MIF induction of COX-2 or other endpoints (data not shown). The induction by MIF of COX-2 protein was significantly inhibited by 30 min pretreatment with the p38 MAP kinase inhibitor SB203580 ($p < 0.05$). In contrast, inhibition of the ERK MAP kinase pathway with PD98059 had no effect on MIF-induced COX-2 protein. Similar effects were observed on COX-2 mRNA as measured by quantitative real-time PCR. Treatment with MIF significantly increased COX-2 mRNA ($p < 0.02$; Figure 2C), and MIF-induced COX-2 mRNA expression was significantly inhibited by p38 MAP kinase inhibition ($p < 0.05$). In contrast, ERK antagonism had no effect on MIF-induced COX-2 mRNA.

These findings were confirmed at the level of PGE_2 release. Unstimulated RA FLS released PGE_2, which was significantly increased by MIF ($p < 0.05$; Figure 3). MIF-induced PGE_2 release was significantly inhibited by 30 min pretreatment with the p38 MAP kinase inhibitor SB203580 ($p < 0.01$). In contrast, inhibition of the ERK MAP kinase pathway with PD98059 had no effect (Figure 3).

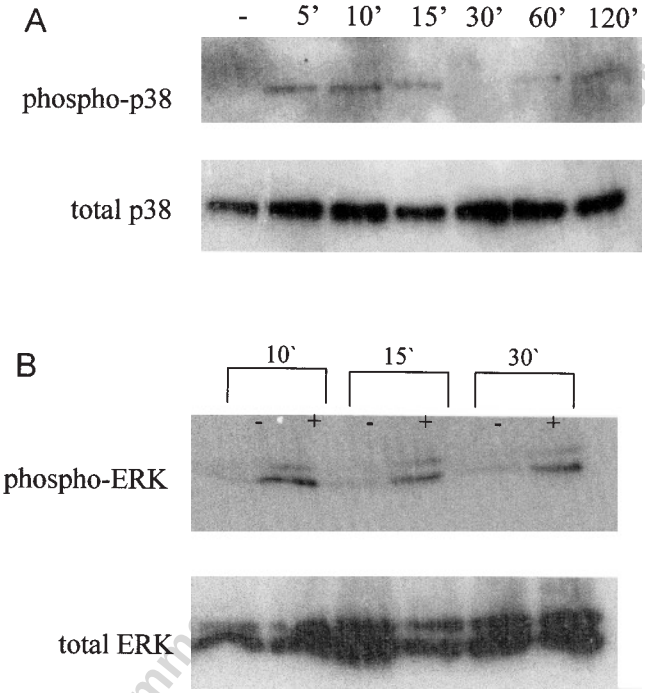

Figure 1. MIF activation of MAP kinases. RA FLS were exposed to recombinant MIF for the indicated times (min) and phosphorylated and total MAP kinase detected using Western blotting with specific mAb. (a) Phosphorylation of p38 MAP kinase was not detected in unstimulated cells but was detected within 5 min of treatment with MIF (100 ng/ml). (b) Phosphorylation of ERK MAP kinase was detectable in unstimulated cells and was increased within 10 min by treatment with MIF (100 ng/ml). Representative illustration from 4 experiments using cells obtained from individual patients with RA.

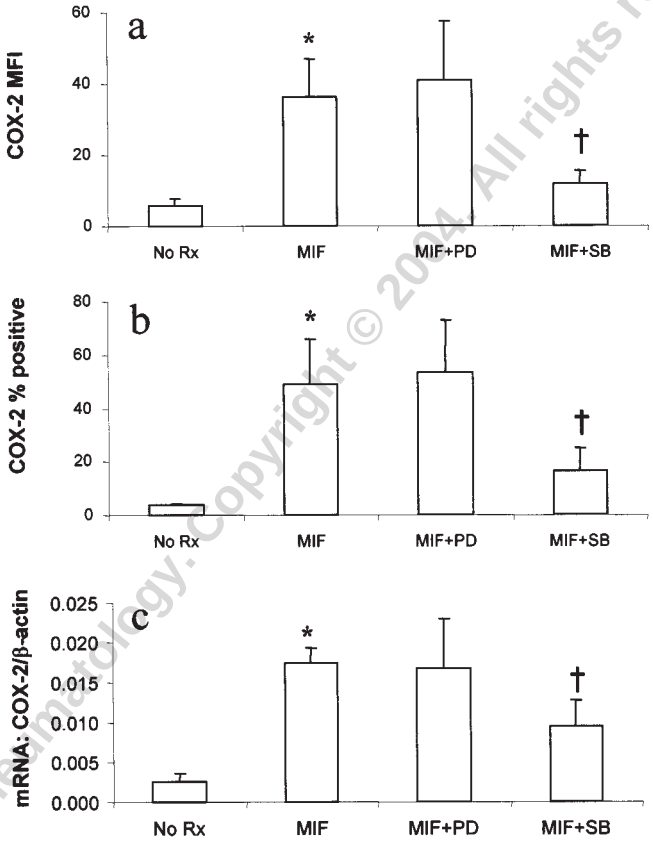

Figure 2. Effect of MAP kinase inhibitors on COX-2 synthesis. Duplicate cultures of RA FLS were exposed to recombinant MIF (100 ng/ml) with or without an inhibitor of the ERK MAP kinase pathway, PD98059, 50 µM (PD) or an inhibitor of p38 MAP kinase, SB203580, 5 µM (SB). Intracellular COX-2 protein content was measured using permeabilization flow cytometry and expressed as (a) mean fluorescence intensity (MFI) or (b) the percentage of cells expressing fluorescence above a gate set with control IgG-labelled cells. COX-2 mRNA was analyzed using real-time PCR (c). MIF induced the expression of COX-2 protein and COX-2 mRNA. Data are mean ± SEM of n = 3 (a, b) or n = 4 (c) experiments using cells obtained from individual patients with RA. *$p < 0.05$ compared to untreated cells; †$p < 0.05$ compared to MIF-treated cells.

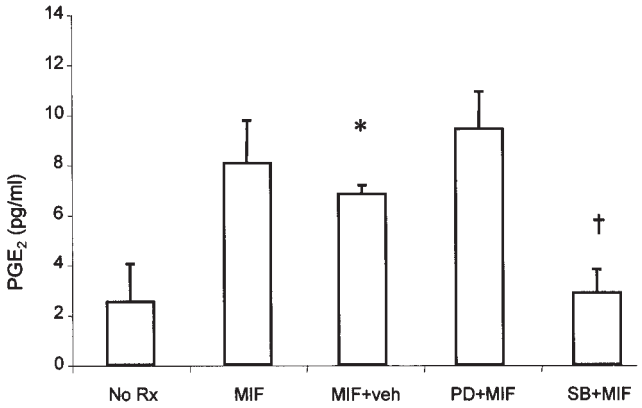

Figure 3. Effect of MAP kinase inhibitors on PGE_2 protein. Duplicate cultures of RA FLS were treated with recombinant MIF (100 ng/ml) with or without the ERK inhibitor PD98059, 50 µM (PD) or the p38 inhibitor SB203580, 5 µM (SB). Supernatants were collected after 8 h and PGE_2 was measured by competitive ELISA. Data are mean ± SEM of 3 experiments using cells obtained from individual RA patients. *$p < 0.05$ compared to untreated cells; †$p < 0.05$ compared to MIF-treated cells.

We next investigated the effect of MIF on FLS IL-6 and IL-8 production. Low level basal release of IL-6 by RA FLS was observed, and this was significantly increased after MIF treatment ($p < 0.05$; Figure 4A). Antagonism of p38 with the p38 MAP kinase inhibitor SB203580 resulted in a significant decrease in MIF-induced IL-6 ($p < 0.05$). In contrast, inhibition of the ERK MAP kinase pathway with PD98059 had no effect. As assessed by quantitative real-time PCR, IL-6 mRNA was also increased by MIF, but neither MAP kinase inhibitor had an effect on MIF-induced IL-6 mRNA (Figure 4B). RA FLS also expressed low basal levels of IL-8, and MIF significantly increased this production ($p < 0.05$; Figure 5). Neither MAP kinase inhibitor had any effect on MIF-induced IL-8.

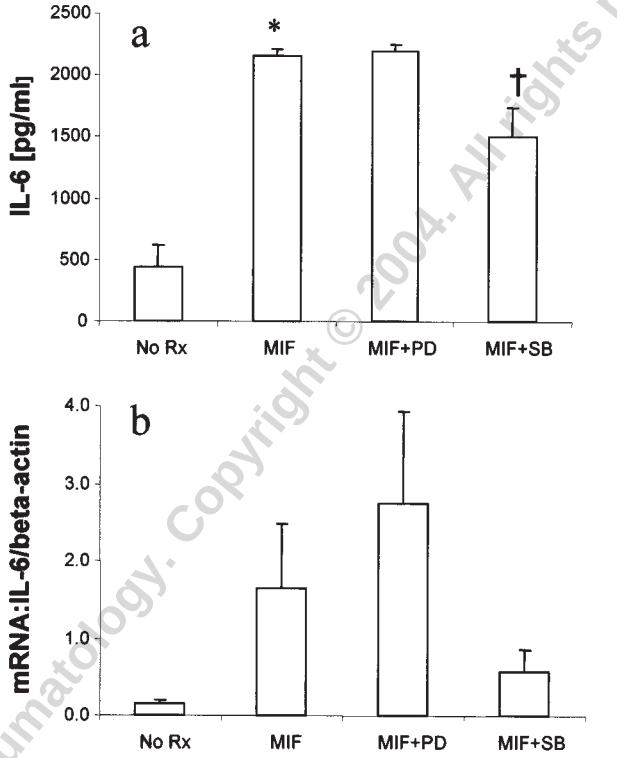

Figure 4. Effect of MAP kinase inhibitors on IL-6 production. Duplicate cultures of RA FLS were treated with recombinant MIF (100 ng/ml) with or without the ERK inhibitor PD98059, 50 µM (PD) or the p38 inhibitor SB203580, 5 µM (SB). IL-6 was measured in culture supernatants by ELISA (a) and IL-6 mRNA was analyzed using real-time PCR (b). Data are mean ± SEM of 3 experiments using cells obtained from individual RA patients. *$p < 0.05$ compared to untreated cells; †$p < 0.05$ compared to MIF-treated cells.

DISCUSSION

Although not as well studied as cytokines such as IL-1 and TNF, MIF has been implicated as a pivotal participant in RA pathogenesis. MIF is produced by T cells, monocytes, and endothelial cells under the influence of lipopolysaccharide (LPS), IL-1, and TNF-α, and like other important upstream cytokines is capable of upregulating the inflammatory activity of these cells[2,23]. MIF is a cytokine with a broad range of roles in innate and adaptive immune responses, including mediation of cutaneous delayed-type hypersensitivity and endotoxic and septic shock[24-26]. Bozza and others have recently shown that mice bearing a targeted disruption of the MIF gene are resistant to the lethal effects of LPS-induced shock[27]. Previous studies described abundant expression of MIF in human RA synovial FLS, macrophages, and T cells, and the overexpression of MIF in RA compared to control tissues[3]. The potential role of MIF in RA is further highlighted by studies in animal models of RA, where anti-MIF therapy resulted in significant inhibition of disease in rat adjuvant arthritis[4], reduced severity in mouse antigen-induced arthritis[5], and delayed onset and decreased frequency in mouse collagen-induced arthritis[28].

We have reported that FLS-derived MIF induces monocyte TNF-α production[3], but the range of effects exerted directly by MIF on FLS activation is only now beginning to be understood. We recently described MIF induction of FLS proliferation *in vitro*[9] and *in vivo*[29]. It has also been reported that MIF is able to induce synoviocyte expression of proinflammatory genes including $cPLA_2$ and COX-2[7] and matrix metalloproteinases[30]. The mechanisms of MIF-induced activation of synoviocyte gene expression are incompletely understood.

Phosphorylation of MAP kinases leads to expression of target genes important in inflammation and proliferation, and understanding this has led to increasing recognition of the potential importance of MAP kinases in RA[8]. Activation of distinct MAP kinase subtype cascades is dependent on the type of cells and stimuli used, and the functional role of each MAP kinase may differ between various cell types. In general, however, the ERK cascade appears to mediate

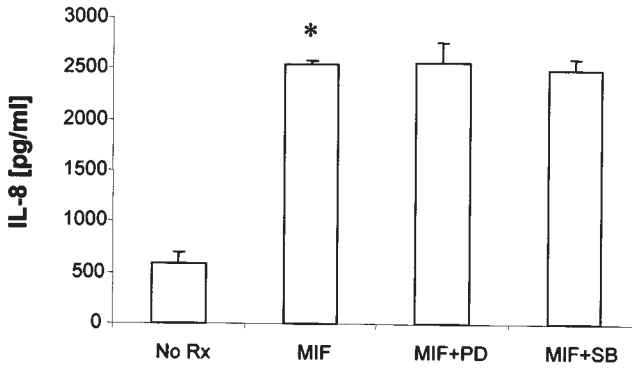

Figure 5. Effect of MAP kinase inhibitors on IL-8 production. RA FLS were treated with recombinant MIF (100 ng/ml) with or without the ERK inhibitor PD98059, 50 µM (PD) or the p38 inhibitor SB203580, 5 µM (SB). Experimental conditions were performed in duplicate, and IL-8 was measured in culture supernatants by ELISA. Data are mean ± SEM of 3 experiments using cells obtained from individual RA patients. *$p < 0.05$ compared to untreated cells; †$p < 0.05$ compared to MIF-treated cells.

signals promoting cell proliferation, differentiation, or survival, whereas p38 MAP kinase appears to be involved in cell responses to stresses and cytokines[8,31]. It has been reported that MIF activates the ERK MAP kinase pathway in murine fibroblasts[32] and rat osteoblasts[33], and we recently confirmed the utilization of ERK MAP kinase by MIF in the regulation of RA FLS proliferation *in vitro*[9]. The potential for MIF to activate p38 MAP kinase, and thus potentially influence synoviocyte gene expression, has not been studied.

Our findings suggest for the first time that MIF is able to induce the phosphorylation of p38 MAP kinase. Activation of p38 MAP kinase was required for MIF-induced COX-2 expression, and ultimately PGE_2 release, by FLS. The importance of the p38 MAP kinase pathway to RA is evidenced by suppression of animal models of RA by specific p38 MAP kinase inhibitors[12,13]. COX-2 expression has been reported to be regulated by the p38 MAP kinase pathway, with effects reported to be particularly dependent upon RNA stabilization[11,14,15]. Our findings suggest a role for the p38 MAP kinase pathway in transcriptional regulation by MIF of COX-2, and thus regulation of PGE_2, in FLS.

No studies on the regulation of synoviocyte cytokine expression by MIF have been reported. IL-6 is a primary product of RA FLS and is perhaps the most abundant cytokine in RA[8]. Our findings reveal that MIF induces FLS expression, and release of IL-6 and MIF induction of FLS IL-6 also requires the participation of the p38 MAP kinase pathway, at least at the translational level. This is in keeping with reports of IL-1-induced IL-6 being dependent on p38 phosphorylation[15,16]. IL-6 is increasingly recognized as an important cytokine in RA, on the basis especially of positive results in human therapeutic trials using an anti-IL-6 receptor monoclonal antibody[18]. Current data suggest the hypothesis that therapeutic antagonism of MIF could be associated with reductions in synovial IL-6.

IL-8 has long been considered to play a role in inflammatory conditions including RA[34,35]. We observed that MIF induces FLS expression of IL-8. Stimulation of this cytokine suggests a mechanism of MIF-dependent synovial chemoattraction. Interestingly, neither ERK nor p38 MAP kinase pathways appear to be utilized by MIF in the induction of IL-8. A possible role for NF-κB in this process is unlikely, as we found MIF was unable to activate this pathway in FLS[9]. IL-8 production by a human epidermal carcinoma cell line has been shown to be SAPK/JNK-dependent[35]. Activation of the SAPK/JNK pathway by MIF should be investigated.

Of note, a reduction in p38 phosphorylation was consistently observed at 30 min after MIF stimulation. This may be explained by the expression of MAP kinase phosphatase (MKP) enzymes, which are capable of dephosphorylating activated MAP kinases by targeting the 2 critical phosphorylation sites in the activation loop of MAP kinase. The genes encoding these proteins are inducible by many of the stimuli that cause MAP kinase activation, suggesting they might be involved in the feedback control of MAP kinase activity[36]. The effect of MIF on MKP activation is currently being investigated.

We also confirm the stimulation of phosphorylation by MIF of ERK in RA FLS, and we have previously reported the dependence on the ERK pathway of MIF-induced FLS proliferation[9]. The involvement of the p38 MAP kinase pathway in MIF-induced COX-2 and IL-6 expression, the ERK kinase-dependence of MIF-induced proliferation[9], and the p38- and ERK MAP kinase-independent stimulation of RA FLS IL-8 suggest that MIF may utilize distinct intracellular signal transduction pathways in the induction of distinct outcomes.

We observed that MIF induces p38 MAP kinase in RA FLS. We also found that MIF induces the expression of RA FLS IL-6 and IL-8; and that RA FLS COX-2, PGE_2, and IL-6 but not IL-8 upregulation are p38-dependent. These findings suggest divergent MAPK utilization by MIF in its stimulation of diverse aspects of FLS activation. The ability of MIF to activate FLS via p38 and ERK MAP kinase pathways independent of NF-κB may be relevant to the glucocorticoid antagonistic effects of MIF.

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