Macrophage Migration Inhibitory Factor Up-regulates Expression of Matrix Metalloproteinases in Synovial Fibroblasts of Rheumatoid Arthritis*

(Received for publication, September 17, 1999, and in revised form, October 19, 1999)

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Neutral matrix metalloproteinases (MMPs) are responsible for the pathological features of rheumatoid arthritis (RA) such as degradation of cartilage. We herein show the up-regulation of MMP-1 (interstitial collagenase) and MMP-3 (stromelysin) mRNAs of cultured synovial fibroblasts retrieved from rheumatoid arthritis (RA) patients in response to macrophage migration inhibitory factor (MIF). The elevation of MMP-1 and MMP-3 mRNA was dose-dependent and started at 6 h post-stimulation by MIF, reached the maximum level at 24 h, and was sustained at least up to 36 h. Interleukin (IL)-1β mRNA was also up-regulated by MIF. These events were preceded by up-regulation of c-jun and c-fos mRNA. Tissue inhibitor of metalloproteinase (TIMP)-1, a common inhibitor of these proteases, was slightly up-regulated by MIF. Similarly, mRNA up-regulation of MMP-1 and MMP-3 was observed in the synovial fibroblasts of patients with osteoarthritis. However, their expression levels were much lower than those of RA synovial fibroblasts. The mRNA up-regulation by MIF was inhibited by the tyrosine kinase inhibitors genestein and herbimycin A, as well as the protein kinase C inhibitors staurosporine and H-7. On the other hand, the inhibition was not seen after the addition of the cyclic AMP-dependent kinase inhibitor, H-8. The mRNA up-regulation of MMPs was also inhibited by curcumin, an inhibitor of transcription factor AP-1, whereas interleukin-1 receptor antagonist, an IL-1 receptor antagonist, failed to inhibit the mRNA up-regulation. Considering these results, it is suggested that 1) MIF plays an important role in the tissue destruction of rheumatoid joints via induction of the proteinases, and 2) MIF up-regulates MMP-1 and MMP-3 via tyrosine kinases, protein kinase C, and AP-1-dependent pathways, bypassing IL-1β signal transduction.

Degradation of extracellular matrix components is often seen as a typical pathological characteristic of rheumatoid arthritis (RA)* and osteoarthritis (OA) (1). The tissue degradation is thought to be largely mediated by neutral metalloproteinases (MMPs) (2–5). MMPs are mainly produced by synovial fibroblasts (6), in which MMP-1 (interstitial collagenase) is considered to be the rate-limiting enzyme in collagenolysis and elicits degradation of collagen types I, II, III, and X (7). Similarly, MMP-3 (stromelysin-1) is capable of degrading various components of the extracellular matrix, including cartilage aggrecan, and types II, IV, IX, and XI collagen (8) and, moreover, has the potential to activate interstitial procollagenase (9, 10) and gelatinase-B (pro-MMP-9) (11). In this context, MMP-1 and MMP-3 are regarded to be responsible in large part for the connective tissue degradation in RA (6).

MMP-1 and MMP-3 are produced by synovial lining cells of fibroblasts and infiltrating macrophages, and their mRNA levels are greater in RA than in OA (12–14). On the other hand, tissue inhibitor of metalloproteinase (TIMP)-1, which is also released from synovial fibroblasts, is a glycoprotein that forms a 1:1 stoichiometric complex with MMP-1 and MMP-3 (15). A number of reports showed that the proteolytic activities of MMPs in connective tissues are regulated by TIMPs (TIMP-1 and TIMP-2), which contribute to the suppression of excessive tissue degradation by MMPs.

Macrophage migration inhibitory factor (MIF) was initially identified as a soluble factor in culture medium of activated T cells (16, 17); however, its precise biological function long remained unelucidated. Following the cloning of MIF cDNA (18), previously unrecognized biological functions of MIF have been revealed. MIF is released as a hormone by the anterior pituitary gland in endotoxic shock (19, 20) and as a proinflammatory cytokine and glucocorticoid-induced immunomodulator mainly produced by macrophages in response to a variety of inflammatory stimuli (21).

In terms of arthritis, it was reported that an anti-MIF antibody suppressed inflammatory responses in a mouse model of type II collagen-induced arthritis (22). We report herein for the first time that MIF up-regulates mRNAs of MMP-1 and MMP-3 in synovial fibroblasts obtained from RA patients. Moreover, we evaluated the signal transduction pathway of MIF with regard to the up-regulation of MMPs. The present results will shed light on the novel pathological mechanism of tissue destruction in rheumatoid joints and should give a further insight into the regulatory mechanism of the production of MMPs by synovial fibroblasts.

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‡‡ The abbreviations used are: RA, rheumatoid arthritis; OA, osteoarthritis; PKC, protein kinase C; AP-1, activator protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1β; MIF, macrophage migration inhibitory factor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinases; IL-1ra, interleukin-1 receptor antagonist; NEAA, nonessential amino acids; FCS, fetal calf serum; MEM, Eagle’s minimum essential medium; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; bp, base pair; TPA, tetradecanoylphorbolacetate; TRE, TPA-responsive element; TNF-α, tumor necrosis factor-α.
Up-regulation of MMP by MIF

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from commercial sources. Collagenase, staurorosine, genistein, and herbimycin A were purchased from Wako (Osaka, Japan); H-7 and H-8 were from Seikagaku Kogyo (Tokyo, Japan); interleukin-1 receptor antagonist (IL-1ra) was from Anapure Biocreative (Beijing, China); Eagle’s minimum essential medium (MEM) was from ICN Biomedicals (Aurora, Ohio); fetal calf serum from HyClone (Logan, UT); nonessential amino acids (NEAA) were from Life Technologies, Inc.; Isogen RNA extraction kit and GenePure were from Nippon Gene (Toyama, Japan); Biotrack MMP-1 assay kit and Hybond N nylon membrane were from Amersham Pharmacia Biotech; Ex-Taq DNA polymerase, DNA random primer labeling kit, and c-fos cDNA probe were from Takara (Kyoto, Japan); curcumin was from Nakarai Tesque (Kyoto, Japan), and pT7 vector was from CLOT-P (Palo Alto, CA). All other chemicals were of analytical grade. cDNA of c-jun was a kind gift from Dr. M. Sakali of the Department of Biochemistry, Hokkaido University School of Medicine. Recombinant human MIF was expressed in Escherichia coli BL21/D3 (Novagen, Madison, WI) and purified as described (23). It contained less than 1 µg of endotoxin per µg of protein as determined by the chromogenic Limulus amoebocyte assay (BioWhittaker, Walkerville, MD).

Synergial Fibroblasts—Synergial fibroblasts were isolated from knee biopsies of patients with RA or OA at the time of the total joint replacement surgery. The study was conducted according to Declaration of Helsinki principles. Synergial tissue was minced and digested in 0.2% collagenase in MEM containing 5% FCS and 100 µM NEAA for 6 h at 37 °C. After centrifugation and washing, cells were resuspended in MEM supplemented with 10% FCS and NEAA in 100-mm culture dishes in a humidified 5% CO2 atmosphere at 37 °C. After 48 h, nonadherent cells were removed, and adherent cells were trypsinized with 0.25% trypsin/EDTA and were successively passaged. Purity of the cells was >95% fibroblast-like cells as confirmed by microscopic analysis.

mRNA Expression of MMP-1, MMP-3, and TIMP-1 in Response to MIF—To examine the effect of MIF on the mRNA expression of MMPs, cultured synergial fibroblasts of RA and OA patients were used with and without passages. After reaching confluence (10–14 days after initial plating), the primary RA and OA fibroblasts (synoviocytes) without passage were rinsed with PBS and challenged with 1, 10, and 100 ng/ml MIF in 10 ml of serum-free MEM containing NEAA for 12 h. To examine the influence of repeated passages in response to MIF in RA synergial fibroblasts, the same procedure was performed on the 3rd- and 7th-passage RA synergial fibroblasts obtained from the same patient. For the time course study, parallel cultures of 3rd-passage RA synergial fibroblasts were used. The cells were harvested and subjected to Northern blot analysis. In all cell culture experiments, 30 µg of polyvinyl alcohol (B) was added to the culture medium.

Northern Blot Analyses—Human complete coding cDNA for human MMP-1 (2.05 kilobase pairs) in a pSP64 vector was purchased from the American Type Culture Collection. The templates of human MMP-3, TIMP-1, IL-1β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA for Northern blot analyses were obtained by reverse transcription-polymerase chain reaction (PCR) from a human cDNA library of human synergial fibroblasts. The preparation of each cDNA was as follows: MMP-3 (1649 bp), sense primer 5′-GTGGTCATCGCCTATCAGTCT-3′ (83–102) and antisense primer 5′-ACACGGATGTCCTCTACATCT-3′ (1712–1731) (GenBank™ accession number X02532); TIMP-1 (535 bp), sense primer 5′-CTGGTGTTATGCCTTTCTCAGACTG-3′ (35–59) and antisense primer 5′-CAGGAAGGAGAAGGTGACTGTCGAGAG-3′ (545–569) (GenBank™ accession number S62825); IL-1β (870 bp), sense primer 5′-ATCTCACTGCAACATCTTTAG-3′ (34–56) and antisense primer 5′-CTGGTATCGAGGCTCTTGGAA-3′ (881–903) (GenBank™ accession number X02532); GAPDH (1024 bp), sense primer 5′-CCGGAATTCTCAAGAGAGCTGCT-3′ (59–78) and antisense primer 5′-CCGGAATTCTCAAGAGAGCTGCT-3′ (1058–1070) (GenBank™ accession number M35319). Each PCR product was separated by agarose gel purification, purified by GenePure, and subcloned into pT7 plasmid vector by TA cloning. The subcloned plasmids were transformed into DH5α competent cells. After amplification, each insert was prepared by restriction enzyme digestion, checked by a sequencing analyzer (Applied Biosystems Inc., 377A), and used as a template for Northern blot analysis.

Total cellular RNA was isolated from RA and OA synergial fibroblasts using an Isogen RNA extraction kit according to the manufacturer’s protocols. RNA was quantitated by spectrophotometry, and equal amounts of RNA (10 µg) from control and test samples were loaded on a formaldehyde-agarose gel. The gel was stained with ethidium bromide to visualize RNA standards, and the RNA was transferred onto a nylon membrane. Fragments obtained by restriction enzyme treatments for MMP-1, MMP-3, TIMP-1, IL-1β, c-jun, c-fos, and GAPDH were labeled with [α-32P]dCTP using a DNA random primer labeling kit. Hybridization was carried out at 42 °C for 24–48 h. Post-hybridization washes were performed in 0.1% SDS, 0.2× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate) at 65 °C. The radioactive bands were visualized by autoradiography on Kodak X-AR5 film and quantitatively analyzed using NIH Image System. Multiple autoradiographic data were examined to ensure that the results reflected those produced in the linear range of the film. The results were standardized with respect to GAPDH mRNA levels. Comparison of ethidium bromide-stained gels with corresponding GAPDH mRNA levels showed that the GAPDH mRNA levels reflected total RNA loaded onto gels.

Preparation of MIF (P1A)—MIF was unexpectedly found to be an isomerase, converting n-2-carboxy-2,3-dihydroxyxindole-5,6-quinone (n-dopachrome) to 5,6-dihydroxyxindole-2-carboxylic acid, in which the N-terminal proline functions as a catalytic base (24, 25). To examine the biological link between isomerase activity and biological functions, cDNA of the P1A mutant of MIF was prepared using a site-directed mutagenesis technique as described previously (26). In brief, the sense primer designed was 5′-CATATGGGCATGTTCATCGTA-3′ and the antisense primer was 5′-GATCTCATGCGCAAGGTTGAGTT-3′ containing the 5′-end sequence encoding an alanine instead of a proline after the initiating methionine with an NdeI restriction site, and the antisense primer was 5′-GATCTCATGCGCAAGGTTGAGTT-3′ containing a 3′-end sequence identical to the wild-type cDNA with a BamHI restriction site. After PCR, the product was subcloned into pT7 vector. The P1A mutant MIF was expressed and purified as described previously (23). The overall protein structure of P1A examined by crystallography was well conserved in comparison with wild-type MIF, consistent with a previous report (25, 27).

Effect of MIF on the Production of MMP—Both 3rd-passage RA and OA synovial fibroblasts were used to investigate the effect of MIF on the protein levels of MMP-1. After reaching confluence (10–14 days after initial plating), the cells were trypsinized and then plated on a 24-well plate dish at the density of 4 × 105 cells per well in 500 µl of MEM containing 10% FCS and NEAA. After 48 h, the medium was replaced with 300 µl of serum-free MEM containing NEAA and various doses of MIF (0, 1, 10, and 100 ng/ml) and 1 and 10 µg/ml. After 48 h, the supernatants were collected and subjected to ELISA for MMP-1. For the time course study, we used a procedure similar to the dose-response study in the presence of 1 µg/ml MIF with regard to the 3rd-passage RA synovial fibroblasts, and we obtained aliquots at the indicated times up to 36 h.

MMP-1 was assayed by a sandwich enzyme-linked immunosorbent assay (ELISA) using a Biotrack MMP-1 assay kit according to the manufacturer’s protocol. The minimal sensitivity of the assay system was 6.25 ng/ml, and good linearity was observed up to 100 ng/ml. This ELISA system, all forms of MMP, including pro-MMP-1, MMP-1, and MMP-1 complexed with TIMP-1, could be measured.

Inhibition of Up-regulation of MMP mRNA by Reagents—To investigate the signal transduction pathways leading to the up-regulation of MMP mRNA by MIF, 3rd-passage RA synovial fibroblasts were used. After reaching confluence, the cells were treated with or without MIF (1 µg/ml) at 30 min after the addition of genistein (0, 10, and 100 µM), staurosporine (0, 1, and 100 nM), and herbimycin (0, 1, 10, and 100 nM) and H-7 (0, 1, and 10 µM), H-8 (0, 1.5, and 15 µM), curcumin (0, 1, and 10 µM), or IL-1ra (0, 10, 100, and 1000 ng/ml) in serum-free medium. After 12 h, the cells were harvested and subjected to Northern blot analysis for MMP-1 and MMP-3 mRNA.

Statistics—Statistical analysis was performed using analysis of variance and Fisher’s Protectant Least Significant Difference as a post hoc test.

RESULTS

Dose-response Study of MIF Effects on MMP, TIMP-1, and IL-1β mRNA Expression of Synovial Fibroblasts—In both primary RA and OA synovial fibroblasts, MMP-1 mRNA was markedly up-regulated in a dose-dependent manner in response to MIF ranging from 1 ng/ml to 10 µg/ml for 12-h treatment (Fig. 1, A and B). With regard to MMP-3, its mRNA level was already high in RA fibroblasts, which might have
and TIMP-1 mRNA was not observed at any dose up to 10 ng/ml MIF in RA synovial fibroblasts, and up-regulation of MMP-1 and MMP-3 mRNA were coordinately expressed in a dose-dependent manner, reaching the maximal level at 10 ng/ml MIF. The mean mRNA levels of MMP-1 and MMP-3 mRNAs were much higher in RA fibroblasts than in OA fibroblasts (Fig. 2). It is of note that TIMP-1 mRNA was markedly expressed in both RA and OA synovial fibroblasts.

**Time Course Study of MMP, TIMP-1, c-jun, c-fos, and IL-1β mRNA Expression in RA Synovial Fibroblasts—**MMP-1 mRNA expression of 3rd-passage RA synovial fibroblasts was found to increase at 6 h post-stimulation with MIF (1 μg/ml) and reached the maximum at 24 h (Fig. 3A). The enhanced mRNA level was sustained for at least 36 h. Similarly, MMP-3 mRNA started to increase at 3–6 h post-stimulation, but the magnitude of the increase was relatively small compared with that of MMP-1. As for TIMP-1, its mRNA was slightly increased by MIF. We found no significant change of mRNA levels in these proteinases in the absence of MIF during the time study. On the other hand, increases of c-jun, c-fos, and IL-1β mRNA levels in response to MIF (1 μg/ml) were observed (Fig. 3B). The c-jun mRNA was up-regulated at 30 min post-stimulation and then gradually decreased. The c-fos mRNA was markedly up-regulated at 30 min post-stimulation and returned to the basal level immediately at 1 h. As for IL-1β mRNA, it started to increase at 3 h post-stimulation, reaching its maximum at 6 h, and gradually decreased.

**Effects of a Mutant MIF (Pro-1→Ala) and Heat-denatured MIF on mRNA Levels of MMPs—**The 3rd-passage RA synovial fibroblasts were used in this study. The potential of MIF for induction of MMP-1 and MMP-3 mRNA was significantly inhibited by heat inactivation at 65 °C for 1 h (Fig. 4). This fact supported our previous data found by nuclear magnetic resonance suggesting that MIF was structurally stable against heat inactivation (23). On the other hand, the P1A mutant MIF, which was prepared by replacing the N-terminal proline with alanine, lost the biological ability to enhance MMP mRNA expression. These results indicated that the mRNA up-regulation of MMPs was MIF-specific and that the N-terminal proline was important for induction for mRNA of MMPs.

**MMP-1 Production by Synovial Fibroblasts in Response to MIF—**MMP-1 protein was detected in all the supernatants of the 3rd-passage RA and OA synovial fibroblasts. In the dose-response study, the MMP-1 level was significantly up-regulated at the doses of more than 1 μg/ml MIF. The mean MMP-1 levels were higher in RA synovial fibroblasts than in OA synovial fibroblasts.

**Effects of MIF on the expression of MMP-1, MMP-3, TIMP-1, and IL-1β mRNA in primary RA and OA synovial fibroblasts.** By using primary synovial fibroblasts (synoviocytes), Northern blot analysis was carried out as described under “Experimental Procedures.” Total RNAs of RA synovial fibroblasts (A) and OA synovial fibroblasts (B) treated with serum-free medium containing various concentrations of MIF for 12 h were subjected to Northern blot analysis, hybridized with 32P-labeled human cDNA probes of MMP-1, MMP-3, TIMP-1, IL-1β, and GAPDH, and visualized by autoradiography. 1st lane, untreated OA synovial fibroblasts; 2nd lane, untreated RA synovial fibroblasts; 3rd lane, OA synovial fibroblasts treated with 1 μg/ml MIF; 4th lane, RA synovial fibroblasts treated with 1 μg/ml MIF; 5th lane, 5 μg/ml MIF; 6th lane, 10 μg/ml MIF.

**Fig. 1.** Effects of MIF on the expression of MMP-1, MMP-3, TIMP-1, and IL-1β mRNA in primary RA and OA synovial fibroblasts.
OA synovial fibroblasts, irrespective of the dose of MIF stimulation. The level of MMP-1 was significantly elevated by the dose of 1 μg/ml MIF in both RA (p < 0.0001, versus control of RA) and OA (p < 0.005, versus control of OA). For the time course study, MMP-1 of RA synovial fibroblasts was elevated at 12 h after MIF stimulation and increased in a time-dependent manner for at least 36 h (Fig. 5B). We established anti-human MIF monoclonal antibodies, and we confirmed that this response was specific for MIF by neutralization using a monoclonal anti-MIF antibody (data not shown). These facts supported the finding that MIF enhanced the synthesis of MMP-1 in RA synovial fibroblasts to a greater extent than in OA synovial fibroblasts, which may reflect the distinct pathological features of these chronic joint diseases. Indeed, we found that MIF content in synovial fluids of RA patients was significantly higher (85.7 ± 35.2 ng/ml, mean ± S.D.) than in those of OA patients (19.5 ± 5.3 ng/ml) and normal controls (10.4 ± 1.1 ng/ml) (28). It is conceivable that MIF, particularly in RA, plays an important role in tissue destruction of rheumatoid joints.

Effects of Protein Kinase Inhibitors on the Expression of MMP mRNA—We examined whether protein kinases were involved in up-regulation of MMPs by using tyrosine kinase inhibitors (genistein and herbimycin A), protein kinase C (PKC) inhibitors (H-7 and staurosporine), and a cyclic AMP-dependent kinase inhibitor (H-8). When these inhibitors were added and cells incubated for 12 h, genistein significantly suppressed the MIF-induced MMP-1 and MMP-3 mRNA expression at
doses of 10–100 μM (Fig. 6). Similarly, herbimycin A suppressed the up-regulation at doses of 1–10 μM (data not shown). H-7 suppressed MMP-1 and MMP-3 mRNA up-regulation at 10 μM (Fig. 7). Staurosporine also suppressed the up-regulation at 100 nM but was not effective at 10 nM (data not shown). On the other hand, H-8 had no inhibitory effect on the up-regulation even at 15 μM (data not shown). These results indicated that activation of tyrosine kinase and PKC might be essential for MMP-1 and MMP-3 mRNA expression by MIF.

Effects of Curcumin and IL-1ra on the Expression of MMP mRNA—We assessed the effects of curcumin, an inhibitor of c-Jun/AP-1, and IL-1ra, an IL-1 receptor antagonist, to examine whether c-Jun/AP-1 and IL-1 were involved in the up-regulation of MMP-1 and MMP-3 mRNA expression. It is known that curcumin inhibits gene expression of c-Jun induced by tetradecanoylphorbolacetate (TPA), but not that of the c-fos gene (29). This inhibitor suppresses the TPA-induced TRE binding activity of AP-1 protein. We found that curcumin suppressed appreciable amounts of MIF-induced MMP-1 and MMP-3 mRNAs at the dose of 10 μM (Fig. 8). On the other hand, IL-1ra did not inhibit the up-regulation of MMPs by MIF even at the dose of 1 μg/ml (data not shown). These results suggested that up-regulation of MMPs by MIF was mediated by activation of c-Jun/AP-1, bypassing the IL-1 signal transduction pathway.

DISCUSSION

It has been reported that mRNA levels of MMP-1 and MMP-3 are expressed coordinately in the synovial lining cells and more in RA fibroblasts than in OA fibroblasts (12–14, 30). The expression of MMPs is regulated at the transcriptional level by various cytokines and other mediators in a positive or negative manner in physiological conditions. Moreover, enzyme activities of MMPs are post-transcriptionally controlled by activation of the latent proenzymes as well as interaction with their specific inhibitors called TIMPs. TIMP mRNA levels are largely similar in RA and OA synovial fibroblasts. Imbalanced production of MMPs and TIMPs in the pathological conditions of both RA and OA is regarded as a critical component to elicit tissue destruction of joints.

In this study, we showed for the first time that mRNAs of MMP-1 and MMP-3 in RA and OA synovial fibroblasts were significantly up-regulated by MIF. It has been reported that biosynthesis of MMP-1 and MMP-3 is up-regulated by TPA and cytokines/growth factors such as IL-1, tumor necrosis factor-α (TNF-α), epidermal growth factor, and platelet-derived growth factor in a variety of cells, including fibroblasts (31–37). In contrast, these two metalloproteinases are down-regulated by transforming growth factor-β, retinoic acid, and dexamethasone (34, 36, 38–40). Recently, we demonstrated that MIF expression was significantly up-regulated by growth factors such as transforming growth factor-β and platelet-derived growth factor (41). These findings indicate that mRNA of metalloproteinases is delicately regulated through a complex mechanism, including growth factors and cytokines.

We also showed that MIF up-regulates IL-1β mRNA in synovial fibroblasts. It was found that TPA-induced MMP-1 expression was mediated by IL-1 in fibroblasts using IL-1ra (42, 43). With regard to MIF in association with IL-1, MIF has the potential to stimulate IL-1β production (44). This fact suggested the possibility that IL-1β endogenously synthesized in response to MIF could mediate the up-regulation of MMPs. However, we found that IL-1ra did not inhibit the up-regulation of MMP-1 and MMP-3 induced by MIF. Therefore, it is conceivable that MIF-induced up-regulation of MMPs might not be mediated by endogenously synthesized IL-1β.

The gene structures of both MMP-1 and MMP-3 contain several regulatory elements in common in their 5'-flanking promoter regions. These include a TATA box, a TPA-responsive element (TRE), i.e., an AP-1-binding site, and also at least one PEA-3/c-ETS-binding site (38, 45, 46). Judging from these facts, it is conceivable that MMP-1 and MMP-3 could be up-regulated in concert through activation of TRE. Indeed, IL-1, TNF-α, and TPA activate AP-1 via up-regulation of fos and jun genes, which leads to induction of MMP-1 and MMP-3 (29, 47–49). Blocking of PKC, an intracellular receptor of TPA, results in the suppression of MMP-3 transcription in response to IL-1 (50). It is also reported that transcriptional factors such as AP-1 or NF-κB are known to be activated within a signal transduction system downstream from the PKC-mediated cascades (51, 52). Considering these facts, including our results, it appears that the signal transduction from MIF to the promoter region of MMPs may be partly mediated by PKC.

On the other hand, tyrosine kinase is another important mediator for the regulation of MMP-1 transcription (53–55). Tyrosine kinase can be classified into the receptor type and nonreceptor type; however, it is not clear which type of tyrosine kinase is involved in the signal transduction for the up-regulation of MMPs by MIF. It was reported that transcriptional activation of MMP-1 by TPA and IL-1β was inhibited by her- bimycin A, but not by tyrphostin A25, an epidermal growth factor receptor tyrosine kinase inhibitor (53). Herbimycin A exerts a selective inhibitory effect on src-related tyrosine kinase (56). Since up-regulation of MMP mRNA by MIF was strongly inhibited by herbimycin A, it is speculated that MIF may mediate its signal transduction via src-related tyrosine kinase. Then how do PKC and src-related tyrosine kinase interact to activate MMP-1 production? src is a substrate for PKC, and phosphorylation of src by PKC is necessary for signal transduction through the β-adrenergic receptor (57). Thus, it is considered that PKC may be initially activated followed by...
activation of src within the MMP-1 transcriptional activation pathway. Taken together, the signal transduction pathway for the up-regulation of MMP genes by MIF might be mediated via a complex system, including PKC, tyrosine kinase, and AP-1.

We also found that the mRNA levels of MMP-1 and MMP-3 were higher in RA synovial fibroblasts than in OA synovial fibroblasts with and without stimulation by MIF. Different responsiveness to cytokines is often seen between these two fibroblasts. For example, IL-1 responsiveness to cytokines is often seen between these two fibroblasts with and without stimulation by MIF. Different responsiveness to cytokines is often seen between these two fibroblasts. For example, IL-1 responsiveness to cytokines is often seen between these two fibroblasts with and without stimulation by MIF. Different responsiveness to cytokines is often seen between these two fibroblasts with and without stimulation by MIF. Different responsiveness to cytokines is often seen between these two fibroblasts with and without stimulation by MIF.

MMP-3 mRNA in the primary RA synovial fibroblasts may have been induced by MIF released from the macrophage subpopulation. Alternatively, decreased responsiveness to MIF for serial-passaged RA synovial fibroblasts was observed. Similar events for the loss of responses to various stimuli in serial-passaged cells have been reported (64, 65). Therefore, the decreased responsiveness might be caused by aging of fibroblasts in vitro.

Next, we demonstrated that mutant MIF, in which the N-terminal proline was substituted by alanine, lost the activity to up-regulate mRNA expression of MMPs. The N-terminal proline of MIF is invariably conserved in all known homologues beyond species, and functions as a catalytic base for isomerase activity. It has been speculated that there might be a biological link between isomerization activity and biological functions of MIF. In support of this, it was demonstrated by protein structural analysis of MIF that its isomerase activity was directly involved in the biological activities represented by neutrophil activation (67). Thus, our present results further support the essentiality of the N-terminal proline for the biological action, i.e. up-regulation of MMPs. At present, however, there is no direct evidence showing the presence of MIF receptors. It should be verified how isomerase activity is linked to the signal transduction on a molecular basis.

In the RA synovial tissues, a large number of infiltrating T cells, in which the CD4/CD8 ratio is higher than that in peripheral blood, have been observed (68, 69). CD4+ T cells are classified as Th1 and Th2 cells in terms of the cytokines they produce. Thus, Th1 cells secrete IL-2, interferon-γ, and TNF-α, whereas Th2 cells secrete IL-4, -5, -6, and -10. Among the T cell-derived cytokines, only TNF-α and IL-10 are reported to stimulate MMP-1 production by fibroblasts (70, 71). It was reported that MIF stimulates macrophages to produce TNF-α (21), which in turn stimulates them to produce MIF. Accordingly, it is considered that MIF may contribute to these events as a critical mediator between T cells and synovial fibroblasts.

**Fig. 7.** Effect of PKC inhibitor on MIF-induced MMP-1 and MMP-3 mRNA expression. RA synovial fibroblasts (3rd passage) were preincubated for 30 min in the presence of H-7. Then they were co-incubated for 12 h with or without 1 μg/ml MIF and subjected to Northern blot analysis. The blots were hybridized with 32P-labeled human cDNA probes of MMP-1, MMP-3, and GAPDH, and visualized by autoradiography.

**Fig. 8.** Effects of an inhibitor of c-Jun/AP-1 on MIF-induced MMP-1 and MMP-3 mRNA expression. RA synovial fibroblasts (3rd passage) were preincubated for 30 min in the presence of curcumin. Then they were co-incubated for 12 h with or without 1 μg/ml MIF and subjected to Northern blot analysis. The blots were hybridized with 32P-labeled human cDNA probes of MMP-1, MMP-3, and GAPDH and visualized by autoradiography.
to regulate MMP-1 production. In addition, we showed that MIF prominently increased IL-1β mRNA levels, and IL-1β also up-regulated MIF mRNA levels in RA synovial fibroblasts. These facts support the idea that MIF plays a key role in the cytokine network of RA synovial tissues.

In summary, we demonstrated that MIF up-regulated mRNA levels of MMP-1 and MMP-3 in RA synovial fibroblasts. This novel function of MIF is indispensable to comprehend the mechanism of the massive joint destruction in RA. In arthritis, an anti-MIF antibody effectively improved the joint inflammation by decreasing circulating anti-collagen type II IgG2a autoantibodies and suppression of the T cell proliferative response to collagen II (22). These facts encourage us to investigate the molecular mechanism of MIF in more detail with regard to the signal transduction system. Moreover, clinical therapy for RA using anti-MIF antibodies or MIF antagonists should be promising as demonstrated by RA model animals (22).

Acknowledgments—We are grateful to Dr. K. Yasuda of the Department of Orthopedics, Hokkaido University School of Medicine; and Dr. S. Matsuno, Dr. M. Minami of Hokkaido Orthopedic Memorial Hospital, and Dr. H. Tanji of Shin Sapporo Orthopedic Hospital for supplementation of the synovial specimens used in this study. We are also grateful to S. Onodera, K. Kaneda, Y. Mizue, Y. Koyama, M. Fujinaga, and J. R. David, J. R. (1966) Proc. Natl. Acad. Sci. U. S. A. 23. Nishihira, J., Kuriyama, T., Sakai, M., Nishi, S., Ohki, S., and Hikichi, K. (1999) Cytokine 11, 163–167.

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