Macrophage Migration Inhibitory Factor Up-regulates Matrix Metalloproteinase-9 and -13 in Rat Osteoblasts

RELEVANCE TO INTRACELLULAR SIGNALING PATHWAYS

Neutral matrix metalloproteinases (MMPs) play an important role in bone matrix degradation accompanied by bone remodeling. We herein show for the first time that macrophage migration inhibitory factor (MIF) up-regulates MMP-13 (collagenase-3) mRNA of rat calvaria-derived osteoblasts. The mRNA up-regulation was seen at 3 h in response to MIF (10 µg/ml), reached the maximum level at 6–12 h, and returned to the basal level at 36 h. MMP-13 mRNA up-regulation was preceded by up-regulation of c-jun and c-fos mRNA. Tissue inhibitor of metalloproteinase (TIMP)-1 and MMP-9 (92-kDa type IV collagenase) were also up-regulated, but to a lesser extent. The MMP-13 mRNA up-regulation was significantly suppressed by genistein, herbimycin A and 4-amino-5-(4-chlorophenyl)-7-(4-buty)pyrazolo[3,4-d]pyrimidine. Similarly, a selective mitogen-activated protein kinase (MAPK) kinase (MEK)1/2 inhibitor (PD98059) and c-jun/activator protein (AP)-1 inhibitor (curcumin) suppressed MMP-13 mRNA up-regulation induced by MIF. The mRNA levels of c-jun and c-fos in response to MIF were also inhibited by PD98059. Consistent with these results, MIF stimulated phosphorylation of tyrosine, autophosphorylation of Src, activation of Ras, stimulation of MMP-13 mRNA up-regulation, and reduction of c-jun transcription. Osteoblasts obtained from calvariae of newborn JunA mice, defective in phosphorylation of c-Jun, showed much less induction of MMP-13 with the addition of MIF than osteoblasts obtained from wild-type or littermate control mice. Taken together, these results suggest that MIF increases the MMP-13 mRNA level of rat osteoblasts via the Src-related tyrosine kinase-, Ras-, ERK1/2-, and AP-1-dependent pathway.

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes, including collagensases, gelatinases, and stromelysins (1–3). Collagensases cleave fibrillar collagens at neutral pH and play an important role in matrix remodeling. Collagenases are largely categorized into three classes: collagensases 1, 2, and 3, which are mainly secreted from fibroblasts and osteoblasts, neutrophils, and breast carcinoma cells, respectively (1, 3). In rodents, rat osteoblasts and rat osteosarcoma cells have the potential to express collagenase 3 such as MMP-13, but not MMP-1 (4–6). With regard to collagenase production, several hormones and cytokines stimulate MMP-1 synthesis in human and MMP-13 in rat osteoblasts (4, 7, 8). From the data available to date, it is considered that most molecules potentially inducing bone resorption often stimulate MMP-1 production in humans and MMP-13 production in rodents. In this context, it is conceivable that MMP-13 may play a pivotal role in bone remodeling in rats.

Macrophage migration inhibitory factor (MIF) was originally identified as a soluble factor in culture medium of activated-T cells (9, 10); however, its precise biological function was largely unknown for nearly 30 years. Following the cloning of human MIF cDNA (11), an array of novel biological functions of this protein has been reported (12, 13). MIF is released as a hormone by the anterior pituitary gland in endotoxin shock (14) and a glucocorticoid-induced immunomodulator released from macrophages in response to a variety of inflammatory stimuli (15). Regarding the potential role of MIF in induction of MMPs, we revealed that MIF could stimulate MMP-1 and MMP-3 mRNA expression in synovial fibroblasts obtained from rheumatoid arthritis patients (16), suggesting its pathological role for direct destruction of joint tissues in autoimmune disease.

In a previous study, we demonstrated that mouse osteoblasts expressed high amounts of MMP-17; however, its pathophysiological role in the bone tissues remains to be elucidated. We herein report for the first time that MIF enhances the MMP-13 mRNA level in osteoblasts obtained from newborn rat calvariae. Following this discovery, we further investigated the signal transduction pathway of MIF in the event of MMP-13 extracellular signal-regulated kinase; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; JNK, c-Jun N-terminal kinase; mAb, monoclonal antibody; MAPK, mitogen-activated protein; MIF, macrophage migration inhibitory factor; NEAA, nonessential amino acid(s); PBS, phosphate-buffered saline; PD2, 4-amino-5-(4-chlorophenyl-7-(4-buty)pyrazolo[3,4-d]pyrimidine; PTH, parathyroid hormone; RBD, Ras-binding domain; TIMP, tissue inhibitor of matrix metalloproteinases; TPA, tetradecanoyl phorbol acetate; TRE, TPA-responsive element.

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¶ The abbreviations used are: MMP, matrix metalloproteinase; AP-1, activator protein 1; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; JNK, c-Jun N-terminal kinase; mAb, monoclonal antibody; MAPK, mitogen-activated protein; MIF, macrophage migration inhibitory factor; NEAA, nonessential amino acid(s); PBS, phosphate-buffered saline; PD2, 4-amino-5-(4-chlorophenyl-7-(4-buty)pyrazolo[3,4-d]pyrimidine; PTH, parathyroid hormone; RBD, Ras-binding domain; TIMP, tissue inhibitor of matrix metalloproteinases; TPA, tetradecanoyl phorbol acetate; TRE, TPA-responsive element.
mRNA up-regulation to elucidate its intracellular mechanism of action.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following materials were obtained from commercial sources. Collagenase, staurosporine, genistein, and herbimycin A were purchased from Wako (Osaka, Japan); H-7 and H-8 from Seikagaku (Tokyo, Japan); cycloheximide, indomethacin, and tyrphostin A25 from Sigma; [32P]ATP and [32P]orthophosphate (8 mCi/ml, carrier-free) from PerkinElmer Life Sciences (Wilmington, DE); Dulbecco's modified Eagle's medium (DMEM) and paraformaldehyde (PFA) from ICN Biomedicals (Aurora, OH); fetal calf serum (FCS) from HyClone (Logan, UT); α-MEM and nonessential amino acids (NEAA) from Invitrogen; Isogen RNA extraction kit and GenePure from Nippon Gene (Toyama, Japan); Hybrid N nylon membrane and ECL Western blotting detection system from Amersham Biosciences, Inc.; horseradish peroxidase-conjugated antibody from Bio-Rad; nonimmune mouse IgG plus Pansorbin cells from Calbiochem-Novabiochem (La Jolla, CA); Ex-Tag DNA polymerase and DNA random primer labeling kit from Takara (Kyoto, Japan); curcumin from Nakarai Tesque (Kyoto, Japan); pT7 vector from CLONTEC (Palo Alto, CA); anti-mouse Src monoclonal antibody (mAb) (GD11; mouse IgG1) and Ras activation assay kit from Upstate Biotechnology (Lake Placid, NY); antibodies for phosphorylated-form mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, from New England Biolabs (Beverly, MA); and anti-phosphotyrosine mAb from Transduction Laboratories (Lexington, KY). All other chemicals were of analytical grade.

Recombinant rat MIF was expressed in Escherichia coli BL21/D3 (Novagen, Madison, WI) and purified as described (18). It contained less than 1% of endotoxin/μg of protein, as determined by the chromogenic Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD).

**Cells**—Rat calvarial osteoblasts were prepared by serial collagenase digestion as described previously (19). In brief, newborn rat calvariae (day 1) were removed from soft tissues and digested with 0.1% collagenase and 0.2% dispase in DMEM containing 100 μM NEAA for 10 min at 37 °C five times (fractions 1–5), of which fractions 3–5 were used. After centrifugation and washing with the medium, cells were resuspended in DMEM supplemented with FCS and chondoly, 10% newborn calf serum (FCS) and 0.2% dispase in DMEM containing 100 μM NEAA (1% final concentration). The cultures were incubated in a humidified 5% CO₂ atmosphere at 37 °C. After 48 h, nonadherent cells were removed, and adherent cells were harvested after treatment with 0.25% trypsin/EDTA and were successively passaged.

Rat synovial fibroblasts were prepared by 0.2% collageanase digestion of rat synovial tissue. The murine osteogenic cell line MC3T3-E1 was purchased from RIKEN Cell Bank (Tsukuba, Japan) and cultured in α-MEM supplemented with 10% FCS. Rat osteosarcoma cell line UM106 was purchased from the American Type Culture Collection (ATCC) (Rockville, MD), and cultured in Eagle's MEM supplemented with 10% FCS and NEAA.

Rat articular chondrocytes were obtained by digestion of cartilage cartilage tissues from the femoral condyle, tibial condyle, femoral head, and humeral head of 8-week-old rats. Rat skin fibroblasts were obtained by in vitro outgrowth from cells of explant cultures of minced skin tissues from newborn rats. All of these cells were cultured in DMEM supplemented with 10% FCS and NEAA.

To examine the effect of MIF on the expression of MMP mRNA, production of MIF protein, phosphorylation of tyrosine, Src kinase activity, MAPK activity, and Ras activation, cultured rat osteoblasts of the third passages were used throughout the experiment. After reaching confluence (10–14 days after initial plating), the osteoblasts were rinsed with phosphate-buffered saline (PBS), serum-starved for 24 h, and challenged with 100 ng/ml, 1 μg/ml, and 10 μg/ml MIF for 6 h in 10 ml of serum-free DMEM containing NEAA. Rat PTH (10−9 m and 10−8 m) and human PTH (1-84) and 1,25(OH)D3 were used as positive controls. Two sets of the third-passage rat synovial fibroblasts, MC3T3-E1 cells, UM106 cells, second-passage rat articular chondrocytes, and second-passage rat skin fibroblasts were used as control cells.

For the time-course study, parallel cultures of rat osteoblasts were treated simultaneously with or without 10 μg/ml MIF, and harvested at indicated intervals after stimulation. Following this, the cells were subjected to Northern blot analysis to evaluate mRNA levels of MMP-13, TIMP-1, and type I (α) procollagen (coll/Iα1) (1), and also to immunoblot analysis and in vitro kinase assay. To exclude possible endotoxin contamination in recombinant MIF, the effect of heat-denatured recombinant MIF treated at 65 °C for 1 h was also assessed. Then neutralizing effect of an anti-MIF monoclonal antibody (IgG1) on MIF-induced MMP-13 mRNA up-regulation was also assessed. The antibody was prepared by immunizing mice with recombinant rat MIF as described elsewhere (20).

To evaluate involvement of c-Jun phosphorylation in the signaling pathway of MIF, calvarial osteoblasts retrieved from newborn JunAA mice were also examined. In JunAA mice, serine residues 63 and 73 of the Jun gene (Jun Jun allele) were substituted with alanine residues (21). JunAA mice were a kind gift from Dr. E. F. Wagner (Research Institute of Molecular Pathology, Vienna, Austria). Calvarial osteoblasts derived from native strain C57BL/6 mice were used as wild-type osteoblasts for positive controls. We also assessed the involvement of c-Fos in the signaling pathways of MIF using calvarial osteoblasts retrieved from newborn mice that were homozygous for the Fos mutation (Fos+/−) and littermate wild type (Fos+/-) mice as controls. Mice that were heterozygous for the Fos mutation (Fos+/−) (B6, 129-Fos+/− female and male mice) were purchased from the Jackson Laboratory (Bar Harbor, ME). After mating, the genotypes of the newborn mice obtained were determined by reverse transcription-polymerase chain reaction (RT-PCR) analysis of the lysed tail from each of them. These murine calvarial osteoblasts were retrieved as primary synovial fibroblasts, and were cultured in α-MEM supplemented with 10% FCS.

**Up-regulation of MMP-9 and -13 in Rat Osteoblasts by MIF**

**Inhibition of MMP-13 mRNA Up-regulation by Reagents**—To investigate the signal transduction pathways regarding up-regulation of MMP-13 mRNA in response to MIF, effects of various reagents on rat osteoblasts were tested. After reaching confluence following serum starvation for 24 h, the cells were challenged with MIF (10 μg/ml) 30 min after the addition of cycloheximide (3 μM), indomethacin (10 μM), genistein (10 and 100 μM), herbimycin A (1 and 10 μM), PP2 (1, 10, and 50 μM), tyrphostin A25 (100 and 10 μM), H-7 (1 and 10 μM), H-8 (1.5 and 15 μM), PD98059 (5 and 25 μM), SB203580 (1, 10, and 20 μM), and curcumin (1 and 10 μM) in serum-free medium. After 6 h, the cells were harvested and subjected to Northern blot analysis for MMP-13 mRNA.

**Northern Blot Analysis**—Rat c-jun cDNA and rat col/Iα (1) cDNA probes were kindly provided by Dr. M. Sakai (Department of Biochemistry, Hokkaido University Graduate School of Medicine) and Dr. M. Shimabana (Department of Microbiology, Showa University School of Pharmaceutical Sciences, respectively). The templates for Northern blot analyses of rat MMP-13, MMP-2, MMP-9, TIMP-1, c-fos, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were obtained by reverse transcription-PCR from a cDNA library of rat synovial fibroblasts. Preparation of each template was as follows: MMP-13 (424 bp), sense primer 5'-GGCGGAGATCTTGAGAAGCTAC-3' (143–165) and antisense primer 5'-TTGTCGACGGAGGAAACCG-3' (547–566) (GenBank® M60616); MMP-2 (217 bp), sense primer 5'-CGCTGACTGAGCAGAGCT-3' (1642–1663) and antisense primer 5'-CACTTTC- CCGAGTTTGCAC-3' (1540–1568) (GenBank® U65666); MMP-9 (280 bp), sense primer 5'-AAGATGTTCTACGTGCCAC-3' (553–571) and antisense primer 5'-AGAGATCTCTACGGGCG-3' (815–832) (GenBank® U24441); TIMP-1 (413 bp), sense primer 5'-CAGATATCGCTGG-1' (994–1017) (GenBank® M17701). Each PCR product was separated by 1% agarose gel, purified by GenePure, and subcloned into a 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 (ABI 377A), and used as a probe for Northern blot analysis.

Total RNA was isolated from rat osteoblasts using an Isogen RNA extraction kit according to the manufacturer’s protocols. RNA was quantitated using a spectrophotometer, and equal amounts of RNA (20 μg) were electrophoresed on a 1.5% 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-13, MMP-2, MMP-9, TIMP-1, col/Iα (1), c-jun, c-fos, and GAPDH were labeled with [α-32P]deoxyctydine triphosphate using a DNA random primer labeling kit. Hybridization was carried out at 42 °C for 24–48 h.
Post-hybridization washing was performed in 0.1% SDS, 0.2% standard saline citrate (SSC) (1× SSC: 15 mM NaCl, 0.015 M sodium citrate) at 65 °C for 15 min. The radioactive bands were visualized by autoradiography on Kodak X-AR5 film and quantitatively analyzed using the NIH Image system. The results were normalized by GAPDH mRNA levels.

**Immune Complex Kinase Assay**—The immune complex kinase assay was performed as described previously (23). In brief, cells were lysed, precleared by incubating with nonimmune mouse IgG plus Pansorbin cells and Protein A-Sepharose 4B overnight. The supernatant was incubated with the anti-mouse Src mAb (10 μg) and then with 20 μl of Protein A-Sepharose 4B for 2 h. These procedures were performed at 4 °C. The beads were incubated in a kinase buffer containing 370 kBq of [γ-32P]ATP at 30 °C for 10 min. Thereafter, the beads were washed, boiled for 5 min, and subjected to SDS-PAGE. The gel was dried, exposed to an imaging plate for 30 min, and analyzed with a BAS2000 imaging system (Fuji Film, Tokyo, Japan).

**Transfection**—Primary osteoblasts were transfected with a recombinant adenovirus carrying a kinase-defective R c-terminal Src family kinase (Csk). The recombinant virus contains the cytomegalovirus immediate early enhancer, chicken β-actin promoter, and rabbit β-globin poly(A) signal and AxCAtesK (K222R), in which the lysine 222 is replaced by an arginine residue, as described previously (24). The control virus Ax1w1 contains no foreign genes. The third-passage rat primary osteoblasts were infected with the recombinant adenoviruses or control virus Ax1w1 at a multiplicity of infection (MOI) of 100 PFU/cell (Fig. 1). The infection was evaluated using a CCK-8 (Dojindo, Kumamoto, Japan) assay after 24 h of infection. Then more than 10 volumes of medium with 10% FCS was added to the cells. After 48 h of the infection, the cells were washed, and treated with MIF (10 μg/ml) for 6 h. Then they were collected and subjected to Northern blot analysis.

**Polymerase Chain Reaction (PCR)**—PCR for the expression of MMP-13 and MAPKs, including ERK1/2, JNK, and p38, were performed as described previously (25). Briefly, rat primary osteoblasts (2.0×10⁶ cells) were serum-starved for 24 h, treated with MIF (10 μg/ml) added indicated intervals, and then solubilized in Laemmli sample buffer, subjected to SDS-PAGE and visualized using the ECL Western blotting detection system according to the manufacturer’s protocol.

**Immunoblot Analysis**—Cell lysates (1×10⁶ cells) were disrupted with a Polytron homogenizer (Kinematica, Lucerne, Switzerland). The protein concentrations of the cell homogenates were quantitated using a Micro BCA protein assay reagent kit. Equal amounts of homogenates were dissolved in 20 μl of Tris-HCl, 50 mM NaCl (pH 6.8), containing 2-mercaptoethanol (1%), sodium dodecyl sulfate (SDS) (2%), glycerol (20%), and bromphenol blue (0.004%), and heated at 100 °C for 5 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described (22) and transferred electrophoretically onto a nitrocellulose membrane. The membranes were blocked with 1% nonfat dry milk in PBS, probed with 1 μg/ml anti-phosphotyrosine mAb or anti-Src mAb, and reacted with the goat anti-mouse IgG Ab coupled with horse-radish peroxidase. The resultant complexes were processed for detection by enhanced chemiluminescence using ECL Western blotting detection system according to the manufacturer’s protocol.

**RESULTS**

**Dose-response Study of MIF Effects on MMP-13, MMP-2, MMP-9, TIMP-1, and col(1)α (1) mRNA Expression of Rat Osteoblasts**—In rat calvaria-derived osteoblasts, up-regulation of MMP-13 mRNA at 6 h after the challenge with MIF was slight at the dose of 1 μg/ml, but it became apparent at the dose of 10 μg/ml (12.8-fold increase compared with the level of nonstimulated control) (Fig. 1a). The induction of MMP-13 by MIF was much higher than that by PTH (10⁻⁸ to 10⁻⁶ M) (2.1-fold increase at the dose of 10⁻⁶ M), a representative physiologic inducer of MMP-13. TPA (10⁻⁶ M), a positive control, most strongly enhanced MMP-13 mRNA (25.2-fold increase at the dose of 10⁻⁶ M). The TIMP-1 mRNA level was also up-regulated, but to a much lesser extent, at doses ranging from 0.1 μg/ml to 10 μg/ml. The type I procollagen (col(1)α (1)) mRNA level was higher at the basal level and was essentially unchanged with addition of MIF. Moreover, we assessed the responsiveness of MMP-2 and MMP-9 mRNA to MIF. MMP-9 mRNA was significantly up-regulated, similar to MMP-13 but to a lesser extent (4.5-fold increase compared with the level of the nonstimulated control at 10 μg/ml MIF), whereas MMP-2 mRNA was slightly increased.

**Up-regulation of MMP-9 and -13 in Rat Osteoblasts by MIF**

To compare expression levels of MMP-13, TIMP-1, and col(1)α (1) among different cell types in response to MIF, rat primary osteoblasts, rat primary synovial fibroblasts, murine MC3T3-E1 cells, and rat UMR-106 cells were examined. MMP-13 mRNA was up-regulated by MIF (10 μg/ml) in rat primary osteoblasts and in rat synovial fibroblasts (Fig. 1b). It was minimally changed in MC3T3-E1 cells and UMR-106 cells, in which the basal expression level of MMP-13 in UMR-106 was very high. On the other hand, TIMP-1 mRNA was slightly up-regulated by MIF in rat primary osteoblasts, but not changed in synovial fibroblasts, MC3T3-E1 cells, or UMR-106 cells. The mRNA level of col(1)α (1) was essentially not affected by MIF stimuli in all these cells. Furthermore, we examined mRNA expression levels in rat articular chondrocytes and rat skin fibroblasts. MMP-13 mRNA was up-regulated in the articular chondrocytes in response to MIF, but not in skin fibroblasts (Fig. 1c). A slight increase of TIMP-1 mRNA in articular chondrocytes was also observed. To assess the structure-specific action of MIF protein, we treated MIF at 65 °C for 1 h. The potential of MIF for induction of MMP-13 mRNA was significantly reduced by the heat treatment (data not shown). We also assessed the neutralizing effect of an anti-MIF monoclonal antibody on MMP-13 mRNA expression. Addition of 50 μg/ml antibody 30 min prior to addition of MIF significantly suppressed the induction of MMP-13 and TIMP-1 mRNAs, though the neutralizing effect was incomplete (Fig. 1d). Nonimmune mouse IgG (IgG1) minimally affected the expression of MMP-13 mRNA. We performed immunoblot analysis of MMP-13 at the protein level to confirm the results regarding the mRNA level. We found that the MMP-13 protein level was increased in response to MIF (10 μg/ml) by 24 h treatment as seen for the PTH (10⁻⁸ M) and TPA (10⁻⁶ M) treatment used as positive controls (Fig. 2).
regulated in response to MIF at 3 h after stimulation and then decreased. The mRNA level of col(I)α(1) was essentially unchanged throughout the time course. Prior to the up-regulation of the MMP-13 mRNA level in response to MIF, transient increases of c-jun and c-fos mRNA levels were observed. The c-jun mRNA was transiently up-regulated at 1 h after stimulation, and then decreased to the basal level at 3 h. The c-fos mRNA was markedly up-regulated at 30 min after stimulation, and immediately returned to the basal level at 1 h. No significant changes of MMP-13 and TIMP-1 mRNA levels were observed in the absence of MIF (data not shown).

**Effects of Various Reagents on Induction of MMP-13 mRNA by MIF**—To examine whether the effect of MIF on rat MMP-13 mRNA levels depended on de novo protein synthesis or was the result of changes in prostaglandin synthesis, rat osteoblasts were treated with MIF in the presence or absence of cycloheximide or indomethacin. Cycloheximide or indomethacin minimally affected the expression of MMP-13 without MIF stimulation (Fig. 4a). When the cells were stimulated with MIF, cycloheximide (3.6 μM) significantly suppressed up-regulation of MMP-13 mRNA (45.8% of the MIF-stimulated level), suggesting that the induction of MMP-13 mRNA required de novo MIF protein synthesis. On the other hand, indomethacin (10
mM) did not suppress the up-regulation of MMP-13 mRNA. This result suggested that MMP-13 induction by MIF was irrelevant to prostaglandin synthesis.

Next, we examined whether protein kinases were involved in up-regulation of MMP-13 mRNA using protein kinase C inhibitors (staurosporine, H-7), a cyclic AMP-dependent kinase inhibitor (H-8), and tyrosine kinase inhibitors (genistein, herbimycin A, PP2, tyrphostin A25). When these inhibitors were added to rat osteoblasts 30 min prior to MIF stimulation (10 µg/ml), staurosporine (10 nM, 100 nM), H-7 (1 µM, 10 µM), and H-8 (1.5 µM, 15 µM) showed no inhibitory effect on up-regulation of the mRNA of MMP-13 (Fig. 4b). On the other hand, genistein (100 µM) and herbimycin A (1 µM, 10 µM) significantly suppressed the MIF-induced MMP-13 mRNA expression (Fig. 4c). Similarly, PP2, a selective inhibitor for Src-related tyrosine kinase (26, 27), markedly suppressed the up-regulation at the dose of 50 µM, whereas tyrphostin A25, an inhibitor for epidermal growth factor receptor-type tyrosine kinase, did not inhibit the induction at doses ranging from 10 to 100 µM (Fig. 4d). These results indicated that MMP-13 mRNA induction by MIF might depend on Src-related tyrosine kinase.

Effect of MIF on the Phosphorylation of Tyrosine—We analyzed levels of tyrosine phosphorylation of intracellular proteins in response to MIF by immunoblot analysis using an anti-phosphotyrosine mAb. MIF treatment resulted in phosphorylation of multiple cellular proteins observed at 60, 34, and 30 kDa in comparison with controls (0 min) (Fig. 5). The enhancement of tyrosine phosphorylation occurred at 1–5 min after stimulation by MIF, and the phosphorylated 60-kDa protein was considered to be an autophosphorylated member of the Src-protein kinase family.

Effect of MIF on the Autophosphorylation of Src—To determine whether MIF could enhance kinase activity of Src, we performed an immune complex kinase assay using cell lysates of serum-starved rat osteoblasts after precipitation with the anti-Src mAb. Autophosphorylation of Src was enhanced at 3 min after stimulation with MIF (10 µg/ml). The phosphorylation level was sustained at least up to 1 h (Fig. 6a). On the other hand, the amount of Src protein obtained in parallel from rat osteoblasts in the presence of MIF was not changed as determined by immunoblot analysis (Fig. 6b). These results suggested that the enhanced autophosphorylation of Src was a result of up-regulation of Src kinase activity, but not of increased Src protein level.

Effect of Dominant Negative Csk Overexpression on the MIF-induced MMP-13 Expression—Src family kinases are known to be negatively regulated by Csk, and overexpression of kinase-defective Csk was reported to cause activation of Src family kinases (24). To investigate the potential involvement of Src family kinases in the induction of MMP-13 expression by MIF stimulation, we assessed the effect of kinase-defective Csk overexpression on MIF-induced up-regulation of MMP-13. Primary osteoblasts were transfected with adenovirus vector pAC-CA Tcsk carrying kinase-defective Csk or with a control vector.
With MIF (10 μg/ml) for 0, 1, 5, 15, and 60 min were subjected to immunoblot analysis using anti-phosphotyrosine mAb (PY-20) as a primary antibody. Arrowheads indicate three proteins that are significantly phosphorylated at tyrosine residues compared with those of controls collected at time 0.

FIG. 5. Effect of MIF on tyrosine phosphorylation of cellular proteins. Cell lysates of rat primary osteoblasts treated with MIF (10 μg/ml) for 0, 1, 5, 10, 15, 30, and 60 min were subjected to immunoblot analysis. The arrow indicates a 60-kDa band corresponding to Src. c, effect of dominant-negative Csk overexpression on the MIF-induced expression of MMP-13. Northern blot analysis was carried out on the cells transfected with recombinant viruses carrying a kinase-defective Csk expression vector (pAx1CATcsk) (K222R) or control vector (pAx1wl) before MIF (10 μg/ml) treatment for 6 h. WT, nontransfected wild-type.

pAx1wl. Overexpression of the mutant Csk was confirmed by immunoblot analysis (data not shown). The basal and MIF-induced MMP-13 mRNA levels were similar in the wild-type and pAx1wl-transfected rat osteoblasts, whereas there was significant enhancement of the MMP-13 mRNA level in the kinase-defective Csk-transfected cells (9.5-fold increase compared with the level of pAx1wl) (Fig. 6c). The data indicated that overexpression of kinase-defective Csk, leading to activation of Src family kinases, could enhance the effect of MIF on MMP-13 expression. This fact also suggested the possibility that MAPK was involved in the signaling pathway of MIF-induced MMP-13 up-regulation.

Effect of MIF on the Activation of MAP Kinases—We performed immunoblot analysis for three distinct MAP kinases to further assess their involvement in the signal transduction of MIF for induction of MMP-13. We found that ERK1/2 was significantly activated in response to MIF, whereas p38 and JNK were not (Fig. 7a). The level of the phosphorylated form of ERK1/2 was significantly enhanced at 15 min by MIF (1.7-fold increase compared with the value of time 0), and returned to the basal level at 1 h, whereas the levels of phosphorylated-JNK and p38 were minimally changed. The enhanced ERK1/2 activity was inhibited by the Src family-specific tyrosine kinase inhibitors PP2 (50 μM) and herbimycin A (1 μM) (Fig. 7b). The results suggested that activation of ERK1/2 by MIF could be regulated downstream of Src family kinases.

Effect of PD98059 and SB203580 on MIF-induced MMP-13 Expression—We further examined whether activation of ERK1/2 was involved in the up-regulation of MMP-13 using a MAPK/ERK kinase (MEK) 1/2-specific inhibitor (PD98059) and p38-specific inhibitor (SB203580). When these inhibitors were added to rat osteoblasts 30 min prior to MIF treatment for 6 h, PD98059 (5 and 25 μM) significantly suppressed MMP-13 up-regulation, whereas SB203580 (1, 10, and 20 μM) showed no inhibitory effect (Fig. 7c). Thus, it is conceivable that MMP-13 mRNA induction by MIF depends on the activation of ERK1/2.

Effect of Curcumin on MIF-induced MMP-13 mRNA Expression—Next, we tested the effects of curcumin, an inhibitor of c-jun/activator protein (AP)-1, to examine whether c-jun/AP-1...
and 25/μM had no inhibitory effect, whereas SB203580 (1 and 10 μM) had a marked inhibitory effect (Fig. 8), suggesting that c-Fos is involved, at least in part, in up-regulation of MMP-13 by MIF. Osteoblasts obtained from wild-type C57BL/6 mice showed marked up-regulation of MMP-13 in response to MIF similar to rat osteoblasts. On the other hand, the induction was significantly reduced in osteoblasts obtained from JunAA mice (3.1-fold increase compared with the level of the nonstimulated control) compared with cells from the littermate control mice (3.8-fold increase compared with the level of the nonstimulated control) (Fig. 10a). The data suggested that up-regulation of MMP-13 by MIF required, at least in part, phosphorylation of c-jun. We also assessed the role of c-Fos in the MMP-13 induction by MIF using Fos knockout mice (Fos+/+). The induction of MMP-13 was reduced in osteoblasts obtained from newborn Fos−/− mice (1.4-fold increase compared with the level of the nonstimulated control) compared with cells from the littermate Fos+/+ mice (3.1-fold increase compared with the level of the nonstimulated control) (Fig. 10b), suggesting that c-Fos is involved, at least in part, in up-regulation of MMP-13 by MIF.

Effect of MIF on the Phosphorylation of c-Jun—We analyzed levels of c-Jun phosphorylation in response to MIF using [32P]orthophosphate labeling of the cells, immunoprecipitation, and SDS-PAGE. MIF treatment resulted in the phosphorylation of c-Jun (Fig. 11). c-Jun phosphorylation was mostly enhanced at 15 min after stimulation by MIF (3.8-fold increase compared with the level at time 0), and decreased at 1 h.

Effect of MIF on the Activation of Ras—To determine whether MIF could enhance activity of Ras, we performed a Ras activation assay using cell lysates of serum-starved rat osteoblasts. The active form of Ras (Ras-GTP) was increased in...
The expression of MMP-13 is regulated at the transcriptional level by various cytokines and other mediators in a positive or negative manner in physiological conditions. In this study, we showed for the first time that MMP-13 mRNA in rat primary osteoblasts was significantly up-regulated by MIF. It has been reported that MMP-13 secreted by osteoblasts plays an important role in bone resorption. In brief, collagenase degrades the osteoid layer covering the surface of bone that results in exposure of calcified bone matrix so that osteoclasts can steadily adhere to the bone surface (29, 30). In this context, the digestion and resorption of osteoid initiates bone resorption. Supporting this notion, bone resorption induced by PTH is strikingly hampered in collagenase-resistant mutant mice (31). Moreover, type I collagen, accounting for 90% of bone matrix produced by osteoblasts, is essential for differentiation of osteoblasts and mineralization of bone matrix (32). Accordingly, an increase of collagenolytic activity accompanied by enhanced degradation of matrix proteins leads not only to enhanced bone resorption, but also to suppression of bone formation.

Enzyme activities of MMPs are also post-transcriptionally regulated by activation of the latent proenzymes as well as interaction with their specific inhibitors called TIMPs (33). Biosynthesis of MMP-13 is up-regulated by hormones/cytokines such as PTH, IL-1, IL-6, retinoic acid, basic fibroblast growth factor, cortisol, leukemia inhibitory factor, oncostatin M, and platelet-derived growth factor-BB in rat osteoblasts or mouse calvariae (6, 8, 12). From this, we needed a large amount of MIF (10 μg/ml, which is extraordinarily high in comparison with other cytokines) (20). Thus, it is speculated that a higher concentration of MIF may be required to elicit its biological function. In fact, we needed a large amount of MIF (10 μg/ml) to activate osteoblasts in this study. To activate synoviocytes obtained from patients with rheumatoid arthritis to stimulate MMP-13 production, 1 μg/ml MIF was required (16). Cellular responsiveness to MIF may change depending on the pathological state, but this needs further evaluation. Although the receptor of MIF has not yet been isolated, it is hypothesized that the requirement for a high dose of MIF may be the result of a small number of MIF receptors, or its low affinity to the MIF ligand. To confirm the specific action of MIF regarding induction of MMP-13, we demonstrated that MIF-induced MMP-13 mRNA up-regulation was suppressed by the anti-MIF monoclonal antibody.

Regarding the signaling pathways of MIF for induction of MMP-13, we found that Src-related tyrosine kinase was profoundly involved in this inductive process. Both herbimycin A and PP2, selective inhibitors for Src-related tyrosine kinase, suppressed up-regulation of the MMP-13 mRNA level, whereas an epidermal growth factor receptor-type tyrosine kinase inhibitor, tyrphostin A25, failed to inhibit the induction. These data indicate that Src-related tyrosine kinase, but not receptor-type tyrosine kinase, is utilized in intracellular signaling in response to MIF. To date, it has been elucidated that Src family kinases consist of nine members, including Blk, c-Fgr, Fyn, Hck, Lck, Lyn, c-Src, c-Yes, and Yrk, and each molecule encoding a cytoplasmic protein-tyrosine kinase is involved in the transmembrane signal transduction. Among the Src family members, c-Src or v-Src is reported to regulate members of
MMP families in various types of cells (45–47). In addition, c-Src is known to contribute to the Ras-MAPK signaling pathway (48), in which tyrosine phosphorylation of Shc, one of the Src substrates binding Grb2 in v-Src-transformed cells, is a potential cause of Ras activation (49). Activation of the Ras-MAPK signaling pathway induces various transcription factors, including c-Jun and c-fos (50). Recently, another MAPK pathway involving Src kinases has been reported (51). These findings suggest that c-Src may transactivate transcription factor AP-1 in several pathways. Our current data showed enhanced autophosphorylation of Src at 3 min in response to MIF, whereas the levels of Src protein were essentially unchanged. In addition, overexpression of the kinase-defective Csk gene up-regulated both the basal and MIF-induced levels of MMP-13 mRNA perhaps via increases of activity of Src family kinase activities. Furthermore, we observed the activation of Ras in response to MIF with a slight delay from Src activation. These facts strongly indicated that MIF might up-regulate MMP-13 mRNA through activation of Src or Src family kinases.

Next, we investigated the involvement of MAP kinases in the induction of MMP-13 in response to MIF. It has been reported that MIF activates ERK1/2 in NIH3T3 fibroblasts (52). Consistent with this report, MIF increased phosphorylated-form ERK1/2; however, two other MAPK members, JNK and p38, were not phosphorylated. Moreover, phosphorylation of ERK1/2 was inhibited by addition of herbimycin A and PP2. Thus, it is conceivable that the activation of ERK1/2 may be located downstream of Src activation in the signaling pathway of MIF. Furthermore, induction of MMP-13 was also inhibited by PD98059, a selective inhibitor of MEK1/2, whereas SB203580, a specific inhibitor of p38, failed to suppress the MMP-13 up-regulation. Based on these data, it is considered that activation of ERK1/2, but not JNK or p38, could be essential for the up-regulation of MMP-13 in response to MIF.

The rat MMP-13 promoter region contains several consensus transcription factor recognition sequences such as C/EBP, Cbfa1, p53, PEA-3, AP-2, and AP-1 (53). On the other hand, the TIMP-1 promoter contains binding sites for AP-1, PEA-3, and Sp-1 (54). As for the MMP-9 promoter region, it has binding sites for AP-1, PEA-3, NFκB, and Sp-1 (55, 56). These findings indicate that MMP-13, TIMP-1, and MMP-9 promoter regions contain two common regulatory elements, i.e., an AP-1 binding site (TRE) and PEA-3. In this context, it is expected that MMP-13, TIMP-1, and MMP-9 could be up-regulated in accordance with activation of TRE or PEA-3. Indeed, AP-1, alone or in concert with Cbfa1, regulates the transcription of MMP-13 in response to platelet-derived growth factor (57), basic fibroblast growth factor (58), and PTH (59). Consistent with these findings, we observed that MIF-induced up-regulation of MMP-13 mRNA was inhibited by curcumin, an inhibitor of c-Jun/AP-1. Furthermore, we found a significant decrease of the MMP-13 mRNA level in osteoblasts obtained from Fos-/- mice compared with osteoblasts from littermate Fos+/- control mice. Because AP-1 is known to be a homodimer or heterodimer of c-Jun and c-Fos, these results strongly indicated an essential role of AP-1 for MMP-13 induction by MIF.

Concerning transcription factors, up-regulation of c-Jun and c-fos mRNA was seen prior to MMP-13 induction by MIF in the time-course study. As for c-fos induction, phosphorylation of Elk-1 was reported to facilitate formation of the ternary complex composed of a serum-responsive factor, SRE and itself (60), to activate transcription. Because the phosphorylation of Elk-1 is caused by ERK1/2, this mechanism may account of MIF-induced c-fos transcription. This notion concerning the signaling pathway is supported by the facts that MIF phospho-

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