# Macrophage Migration Inhibitory Factor Upregulates Angiogenic Factors and Correlates with Clinical Measures in Rheumatoid Arthritis

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*ABSTRACT.* ***Objective.*** To investigate the relationship between macrophage migration inhibitory factor (MIF) levels and clinical measures in rheumatoid arthritis (RA), and the potential for regulation of angiogenesis in RA.

***Methods.*** Serum and synovial fluid (SF) levels of MIF and vascular endothelial growth factor (VEGF) in patients with RA were determined by sandwich ELISA, and the relationships among MIF, VEGF, and RA clinical measures were analyzed. RA synovial fibroblasts were cultured with recombinant human MIF (rhMIF) and the production of VEGF and interleukin 8 (IL-8) were measured in the conditioned media. The angiogenic effect of MIF was examined using established measures of angiogenesis *in vitro*.

***Results.*** Erythrocyte sedimentation rate, C-reactive protein, and the daily dosage of oral prednisolone were correlated with SF levels of MIF. The SF levels of MIF were found to be higher in patients with bony erosion than in those without (69.2 ± 11.4 ng/ml vs 44.0 ± 6.2 ng/ml; $p = 0.045$). MIF levels had good correlation with VEGF levels ($r = 0.52$, $p < 0.001$ in sera, and $r = 0.6$, $p < 0.001$ in SF). Production of the angiogenic factors VEGF and IL-8 was enhanced in cultured RA synovial fibroblasts stimulated by rhMIF. Endothelial tube formation was augmented when the endothelial cells were cultured with the conditioned media from rhMIF-pretreated SF mononuclear cells, and this phenomenon was reversed by anti-VEGF antibody.

***Conclusion.*** SF MIF may reflect the clinical activity in patients with RA, and rhMIF induces the angiogenic factors in RA synovial fibroblasts. These results suggest that MIF may be an important cytokine in the perpetuation of the angiogenic and inflammatory processes in patients with RA. (First Release April 1 2007; J Rheumatol 2007:34:927–36)

*Key Indexing Terms:*
MACROPHAGE MIGRATION INHIBITORY FACTOR RHEUMATOID ARTHRITIS
VASCULAR ENDOTHELIAL GROWTH FACTOR CLINICAL MEASURES ANGIOGENESIS

Rheumatoid arthritis (RA) is a multisystemic autoimmune disease characterized by persistent synovial inflammation, hyperplasia, angiogenesis, and bony erosion, resulting in joint destruction. Many cytokines play a central role in the pathogenesis of RA, and macrophage migration inhibitory factor (MIF) is known to be one of the proinflammatory cytokines involved in the inflammatory responses and the destructive processes of RA[1,2]. MIF activates T cells and promotes the release by macrophages of proinflammatory mediators such as tumor necrosis factor (TNF), interleukin 1 (IL-1), IL-6, and IL-8, and promotes the interferon-γ-(IFN-γ)-induced production of nitric oxide (NO) by mouse macrophages in an autocrine and a paracrine manner[1,3-6]. All of these roles of MIF are important in the pathogenesis of RA inflammation. Also, MIF activates synovial fibroblasts to produce cytoplasmic phospholipase $A_2$, cyclooxygenase (COX) 2, and matrix metalloproteinase (MMP)-1 and MMP-3[7,8], which suggests that MIF may contribute to the tissue destructive processes of RA. Moreover, MIF also upregulates MMP-13 mRNA in rat

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osteoblasts, indicating that it is involved in collagen matrix degradation and in bone remodeling[9]. It is abundantly expressed by fibroblast-like synoviocytes and macrophages in RA synovium and upregulates the release of TNF-α and IL-1ß, which suggests that it acts as an upstream member of the network of cytokines operating in RA[1]. Recent data suggest that MIF may regulate RA synovial hyperplasia by acting directly and indirectly via the involvements of TNF-α and IL-1ß[10]. In addition to RA, in hepatocellular carcinoma and esophageal cancer, MIF is overexpressed in serum and the tumor tissues of patients, and this overexpression reflects disease progression and prognosis[11,12].

MIF also induces angiogenesis, as it upregulates vascular endothelial growth factor (VEGF) and IL-8 production and stimulates endothelial tube formation[13]. As well as *in vitro*, MIF also induces angiogenesis in the rat cornea *in vivo*[14]. VEGF is a representative angiogenic molecule that stimulates endothelial cell proliferation, angiogenesis, and capillary permeability[15], and it is highly expressed in the inflammatory synovium, synovial fluids (SF), and sera of patients with RA[16]. Moreover, VEGF level is related to markers of RA disease activity such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and tender/swollen joint counts[17]. MIF is known to induce angiogenesis via upregulation of VEGF in some malignant cells[11,12,18-20], but its role in angiogenesis in RA synovial tissues has not been resolved.

We investigated elevated MIF levels in the sera and SF of RA patients and compared them with controls; in addition, we investigated correlations between MIF levels and the clinical variables of RA disease activity. We determined MIF may have potential for the regulation of angiogenesis in RA.

## MATERIALS AND METHODS

*Patients and clinical measures*. The sera of 72 patients with RA and the SF of 45 patients with RA were studied. Synovial samples were obtained from knee joints by diagnostic or therapeutic arthrocentesis and stored at –20˚C. Patients with RA all met the American College of Rheumatology 1987 revised criteria for the classification of RA[21]. Most sera of RA were obtained at the same time as SF. Sera of 15 patients with osteoarthritis (OA) and 31 healthy volunteers, and SF of 49 patients with OA were used as controls. Informed consent was obtained from all patients and healthy volunteers before the study, and the study was approved by the Ethical Committees in Kang-Nam St. Mary's Hospital.

ESR, CRP, and IgM rheumatoid factor titers were determined using standard laboratory methods. HLA-DR typing was performed by polymerase chain reaction (PCR) SSCP, as described[22]. Current dosages of oral prednisolone and the radiological findings of affected joints were evaluated when sera and SF were obtained. A radiologist with no clinical information on the patients determined the presence of bony erosions by reviews of radiographs of both hand joints.

*Measurement of MIF, VEGF, and IL-8*. Concentrations of MIF, VEGF, and IL-8 in sera, SF, or conditioned media were measured by sandwich ELISA, as described below; 0.4 μg/ml of antibodies to human MIF or 4 μg/ml of antibodies to human VEGF and IL-8 (R&D Systems, Minneapolis, MN, USA) was added to a 96-well plate (Nunc, Roskilde, Denmark) and incubated overnight at 4˚C. After treating with blocking solution (phosphate buffered saline, PBS) containing 1% bovine serum albumin (BSA) and 0.05% Tween 20 for 2 h at room temperature, test samples and the standard recombinant MIF, VEGF, and IL-8 (R&D Systems) were added to the 96-well plate and incubated at room temperature for 2 h. After washing 4 times with PBS containing Tween 20, 200 ng/ml of biotinylated MIF or 400 ng/ml of biotinylated VEGF and IL-8 polyclonal antibodies to human cytokines (R&D Systems) were added and the reactions were allowed to proceed for 2 h at room temperature. After washing, 2000-fold diluted streptavidin-alkaline-phosphate (Sigma Bioscience, St. Louis, MO, USA) was added, and the reaction was again allowed to proceed for 2 h. After washing 4 times, 50 μl of diluted avidin peroxidase (1:2000 in diluent) was added. After incubation for 2 h at room temperature, tetramethyl benzidine (TMB) substrate solution (Kirkegaard & Perry Laboratories, Guildford, UK) was added to each well (50 μl) and incubated for 20 to 30 min. Initially the reaction produced a blue color that was monitored by absorbance at 595 nm with a microplate reader (MRX Revelation, Dynex Technologies, Chantilly, VA, USA). When the desired intensity was reached [< 0.8 optical density (OD)], sulfuric acid (2.0 mol/l) was added to each well (50 μl) to stop the color-generating reaction. An automated microplate reader (Vmax, Molecular Devices, Palo Alto, CA, USA) set at 450 nm was used to measure the OD. The sensitivity limit was 15.6 pg/ml for MIF, VEGF, and IL-8. Recombinant human cytokines diluted in culture medium were used as a calibration standard, ranging from 10 to 2000 pg/ml. A standard curve was drawn by plotting OD versus the log of the concentration of recombinant cytokine.

*Isolation of CD3+, CD14+, and CD19+ cells from PBMC and SFMC*. Peripheral blood mononuclear cells (PBMC) and SF mononuclear cells (SFMC) were prepared from heparinized tubes by Ficoll-Hypaque (SG1077) density gradient centrifugation. Anti-CD3, anti-CD14, and anti-CD19 microbeads were used essentially as recommended by the manufacturer (Miltenyi Biotec, Auburn, CA, USA)[23]. PBMC and SFMC were resuspended in 80 μl fetal bovine serum (FBS) staining buffer. Anti-CD3, anti-CD14, or anti-CD19 microbeads (20 μl) were added and incubated for 15 min at 6˚–12˚C. Saturating amounts of fluorochrome-conjugated antibodies were added for another 10 min. Cells were diluted in 2.5 ml 2% FBS staining buffer, pelleted, and resuspended in 500 μl and then separated magnetically, usually on an AutoMACS magnet fitted with a MACS MS column. Flowthrough and two 1-ml washes were collected as the negative fraction. Enriched cells were collected in two 0.5-ml aliquots from the column after removal from the magnet. The purity of cells was assessed by flow cytometric analysis of stained cells on a FACS Vantage sorter. Most of the isolated cells (> 95%) had the specific cell markers for CD3, CD14, or CD19.

*MIF production by anti-CD3 antibody in whole cells, T cells, or non-T cells*. Whole cells, from SFMC and PBMC, were divided into T cells and non-T cells. 1 ml of cell suspension was dispensed into 24-well multiwell plates (Nunc) in 1 ml serum-free RPMI 1640/insulin-transferrin-selenuim A (Life Technologies, Rockville, MD, USA), and the culture plates were incubated 48 h in the presence or absence of 10 μg/ml of anti-CD3 antibody (Pharmingen, San Diego, CA, USA). The culture supernatants were collected and stored at –20˚C until assayed. All cultures were set up in triplicate.

*MIF production by lipopolysaccharide (LPS) and CpG in CD14 and CD19 cells from PBMC*. Whole cells from PBMC were divided into CD14 and CD19 cells. 1 ml of cell suspension was dispensed into 24-multiwell plates (Nunc) in 1 ml serum-free RPMI 1640/insulin-transferrin-selenuim A (Life Technologies), and the culture plates were incubated for 48 h in the presence or absence of 100 ng/ml of LPS (Sigma) or 5 μM of CpG (Life Technologies). The conditioned media were collected and stored at –20˚C until assayed. All cultures were set up in triplicate.

*Isolation of synovial fibroblasts*. Synoviocytes were isolated by the enzymatic digestion of synovial tissues obtained from patients with RA and OA undergoing total joint replacement surgery. The tissues were minced into 2–3 mm pieces and treated for 4 h with 4 mg/ml of type I collagenase (Worthington Biochemical, Freehold, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM) at 37˚C in 5% $CO_2$. Dissociated cells were then centrifuged at 500 g, resuspended in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, and plated in 75 $cm^2$ flasks. After overnight culture, the nonadherent cells were

removed and the adherent cells were cultivated in DMEM supplemented with 20% FCS. The cultures were kept at 37˚C in 5% $CO_2$, and the medium was replaced every 3 days. When the cells approached confluence, they were passed after 1:3 dilution with fresh medium. Synoviocytes from passages 4–8 were used in each experiment. The cells were morphologically homogeneous and exhibited the appearance of synovial fibroblasts, with typical bipolar configuration under inverse microscopy. The purity of the cells ($1 \times 10^4$) was tested by flow cytometric analysis using PE-conjugated anti-CD14 (Pharmingen) and fluorescein isothiocyanate-conjugated anti-CD3 or anti-Thy-1 (CD90) monoclonal antibodies (Pharmingen). At passage 4, most cells (> 95%) expressed the surface markers for fibroblasts (Thy-1), whereas 3.5% of cells were CD14+ and < 1% of cells were CD3+.

*MIF production by various cytokines in synovial fibroblasts*. A homogenous population of synovial fibroblasts from passages 4 to 8 was used for each experiment. The synovial fibroblasts were seeded in 24-well plates at 4 x $10^4$ cells per well in DMEM. Subsequently, 10 ng/ml of IL-1ß (Endogen, Woburn, MA, USA), TNF-α (PeproTech, London, UK), IL-17 (R&D Systems), and CD40L (R&D Systems) were added into the media and the cultures were incubated for 48 h. After 48 h incubation (unless otherwise stated), cell-free media were collected and stored at –20ºC until assayed using ELISA.

*VEGF and IL-8 production by MIF in synovial fibroblasts*. A homogenous population of synovial fibroblasts from passages 4 to 8 was used for each experiment. Synovial fibroblasts were seeded in 24-well plates at $4 \times 10^4$ cells per well in DMEM. Subsequently, various concentrations (1–100 ng/ml) of human MIF, which was produced in native sequence from a recombinant expression[24], were added into the media and the cultures were incubated for 48 h. After 48 h incubation (unless otherwise stated), cell-free media were collected and stored at –20ºC until assayed using ELISA.

*Tube formation assay*. Two hundred fifty microliters of growth factor-reduced Matrigel (10 mg protein/ml) was pipetted into each well of a 24-multiwell culture plate and polymerized for 30 min at 37˚C. Human umbilical vein endothelial cells (HUVEC) that were incubated in M199 with 1% FBS for 6 h or overnight were harvested after trypsin treatment, resuspended in M199 with 1% FBS, loaded onto a layer of Matrigel at a density of 1 x $10^5$ cells/well, and stimulated with MIF (10 ng/ml). Matrigel cultures were incubated at 37˚C and photographed with a digital camera (x40) at 14 or 18 h. Six random fields were measured for each well and the total length of the tube network for each field was calculated using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Each dose was assayed in duplicate.

*Statistical analysis*. Data are expressed as mean ± standard error (SEM). Statistical analysis was performed using Mann-Whitney U-test for the independent samples and Wilcoxon signed-rank test for the related samples. Correlation coefficients were determined by Spearman's rank correlation test. P values less than 0.05 were considered significant.

## RESULTS

*Serum and SF levels of MIF in RA*. Clinical characteristics of the patients with RA were as follows: mean age 49.6 ± 1.3 years, mean disease duration 68.5 ± 6.3 months, ESR 37.7 ± 2.9 mm/h, CRP 2.2 ± 0.3 mg/dl, and current prednisolone dose 4.8 ± 0.3 mg/day. To determine serum and SF levels of MIF in RA, we measured serum and SF levels of MIF from 72 patients with RA and 49 patients with OA (mean age 62.6 ± 1.4 yrs), and serum MIF levels of 31 healthy controls (mean age 47.1 ± 2.1 yrs) by sandwich ELISA. The serum MIF level was higher in patients with RA than in either patients with OA (12.1 ± 1.3 ng/ml vs 7.83 ± 1.5 ng/ml; p = 0.039) or controls (1.7 ± 0.6 ng/ml; p < 0.001), and it was also higher in the SF of patients with RA than in patients with OA (53.52 ± 6.5 ng/ml vs 16 ± 2.8 ng/ml; p < 0.001; Figure 1). Serum and SF levels of MIF had no correlation with age and sex, and SF levels of MIF did not correlate with serum levels (r = 0.38, p = 0.1).

*Relationship between MIF levels and RA clinical variables*. We evaluated whether serum and SF levels of MIF were related to the clinical measures of RA. ESR and CRP were found to correlate with SF levels of MIF (r = 0.34, p = 0.046 and r = 0.38, 0.025, respectively), but not with serum levels of MIF (Figures 2A, 2B). Moreover, ESR and CRP were found to correlate with the current daily dosage of oral prednisolone (r = 0.35, p = 0.03 and r = 0.44, p < 0.001, respectively), which may be consistent with the dosage of oral prednisolone reflecting RA disease activity in some degree. The daily

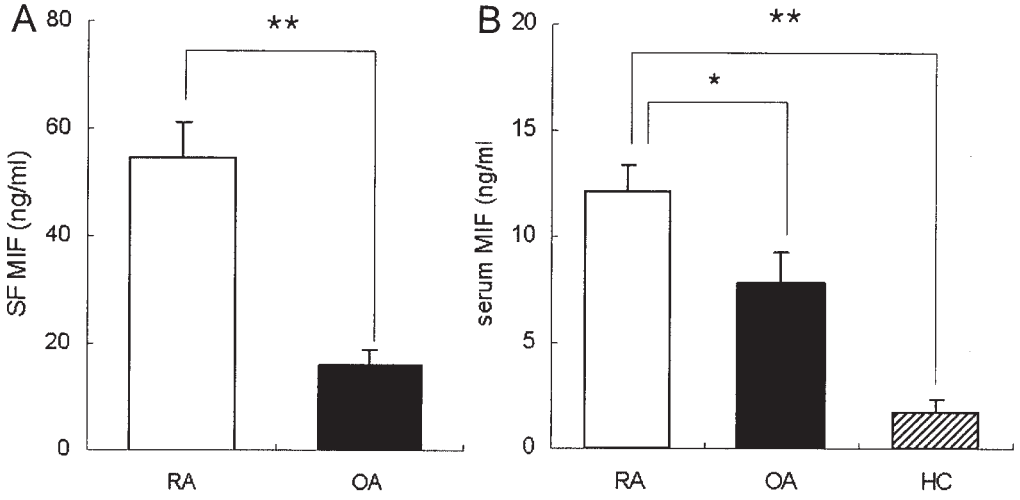



*Figure 1*. A. Concentration of synovial fluid (SF) macrophage migration inhibitory factor (MIF) in patients with RA and OA. B. Serum concentration of MIF in patients with RA and OA and healthy controls (HC). Serum and SF levels of MIF were significantly higher in patients with RA than in controls. *p < 0.05; **p < 0.001.

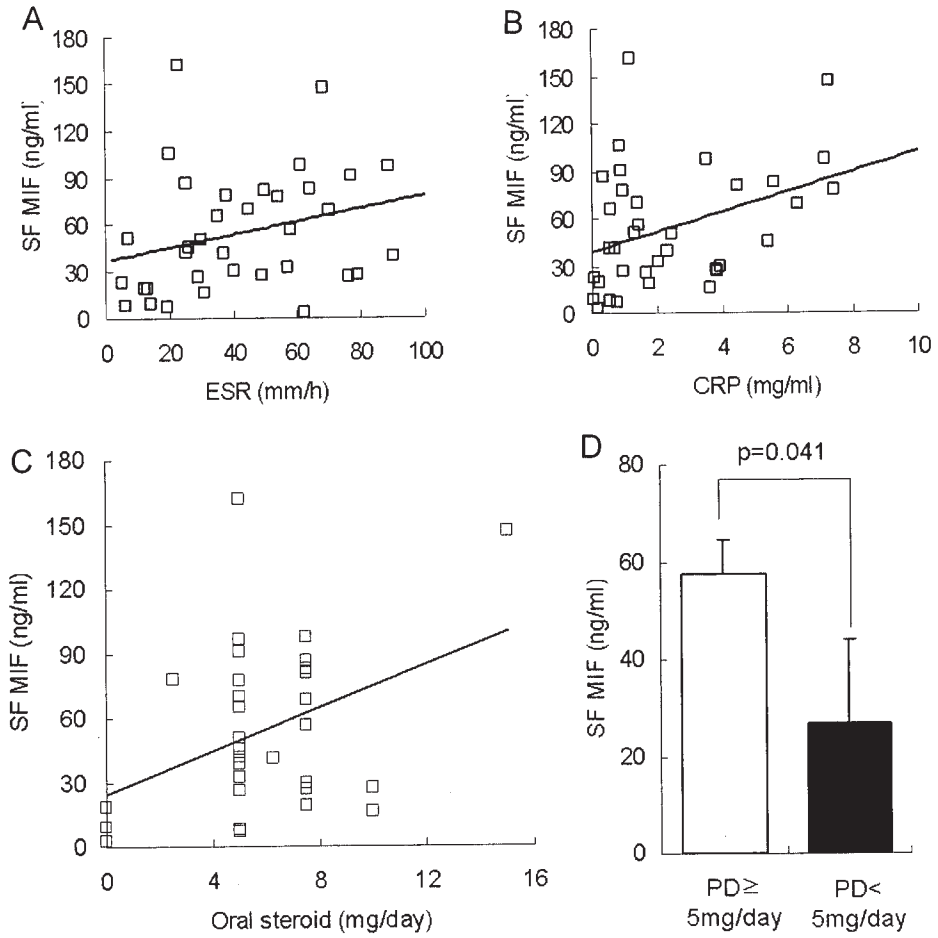
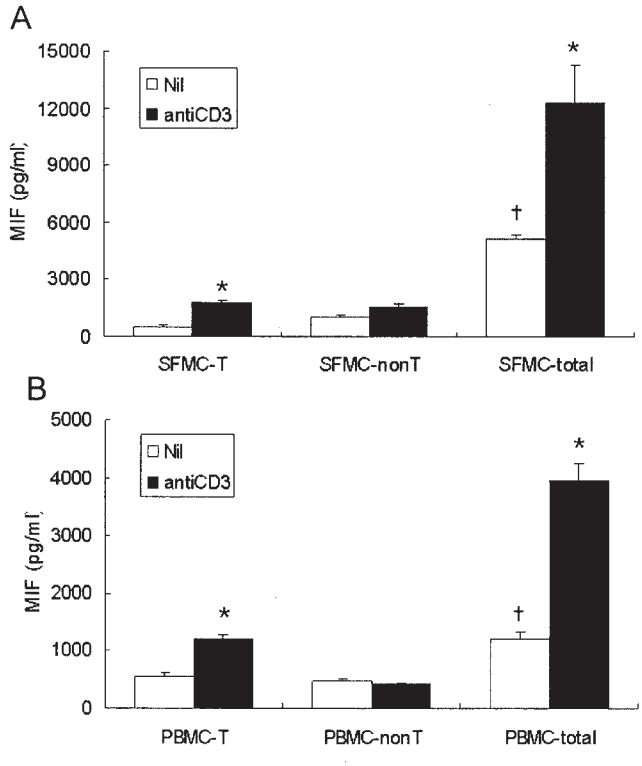



*Figure 2*. Relationships of ESR, CRP, and SF MIF levels in patients with RA. Both ESR (A) and CRP (B) correlated with SF MIF levels (r = 0.34, p = 0.046 and r = 0.38, p = 0.025, respectively). C. Relationship between the current dosage of oral prednisolone and SF MIF levels. Daily dosage of prednisolone was positively correlated with SF MIF levels (r = 0.38, p = 0.029). D. Comparison of SF MIF levels in patients taking ≥ 5 mg/day and < 5 mg/day oral prednisolone (PD) at the time samples were obtained. Patients taking PD ≥ 5 mg/day tended to have higher SF MIF level (57.6 ± 7.1 ng/ml vs 27.1 ± 17.3 ng/ml; p = 0.041).

dosage of oral prednisolone also was found to be positively correlated with SF levels of MIF (r = 0.38, p = 0.029) and patients who took prednisolone ≥ 5 mg/day tended to have a higher level of MIF than those who took < 5 mg/day (57.6 ± 7.1 ng/ml vs 27.1 ± 17.3 ng/ml; p = 0.04; Figures 2C, 2D). SF levels of MIF were higher in patients with bony erosion by plain radiograph than those without (69.2 ± 11.4 ng/ml vs 44.0 ± 6.2 ng/ml; p = 0.045). On the other hand, the serum and SF levels of MIF were not different between groups with positive IgM rheumatoid factor and those without. No difference in serum and SF levels of MIF was found between patients with shared epitopes (HLA*0101, *0401, *0404, or *0405) or those with extraarticular manifestations and those without. The numbers, duration, and kind of disease modifying antirheumatic drug therapy were found to have no correlation with the levels of MIF.

*MIF production by mononuclear cells from peripheral blood and SF.* To determine the main sources of MIF in patients with RA, peripheral blood and SF were obtained from patients with RA and the mononuclear cells were isolated. From the isolated mononuclear cells, T cells and the other cells (non-T cells) were divided using MACS separation methods. Both groups of the cells released MIF constitutively, but anti-CD3-activated T cells produced MIF by more than 2–3 times. Moreover, MIF production was increased greatly after activated T cells were cocultured with the non-T cells (Figures 3A, 3B). T cells produced a similar degree of MIF constitutively regardless of the sites where cells were collected (MIF production of T cells from SF 517.3 ± 69.2 pg/ml and from peripheral blood 551.3 ± 57.9 pg/ml), but non-T cells from SF produced more MIF than those from peripheral blood (MIF production of non-T cells from SF 1016.0 ± 104.1 pg/ml and of non-T cells from peripheral blood 476.5 ± 50.6 pg/ml). However, when T cells were activated by anti-CD3 and cultured with non-T cells, the cells from SF produced far more MIF than the cells from periph-



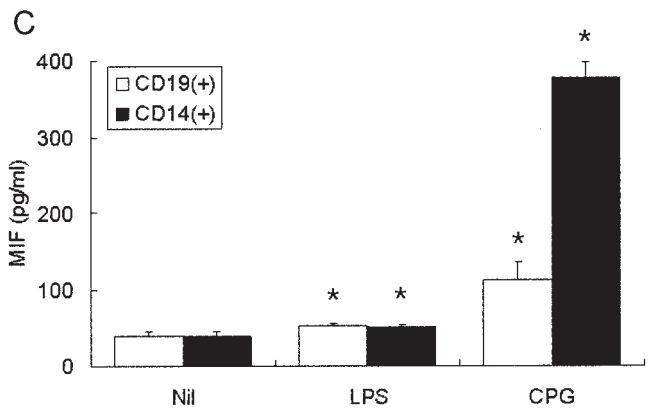




*Figure 3*. Production of MIF by T cells and non-T cells from peripheral blood (PB) and synovial fluid (SF). PB and SF mononuclear cells (MC) were isolated and divided into T cells and other cells (non-T cells). A. Both T cells and non-T cells from SF released MIF constitutively, and the activated T cells produced more MIF. But MIF production was greatly increased after anti-CD3-activated T cells were cocultured with the non-T cell groups. B. Both T cells and non-T cells from PB released MIF constitutively, and again activated T cells produced more MIF, and MIF production was greatly increased after anti-CD3-activated T cells were cocultured with the non-T cell groups. C. PBMC were divided into CD19+ B cells and CD14+ monocytes, and stimulated with 10 $\mu$g/ml lipopolysaccharide (LPS) and 5 $\mu$M CpG. After CpG stimulation, CD14+ cells produced more MIF than CD19+ B cells. *$p < 0.05$ compared with MIF production of unstimulated cells (Nil), and †$p < 0.001$ compared with MIF production from both T cells and non-T cells.

eral blood (cells from SF 12,005.0 ± 1607.8 pg/ml vs cells from peripheral blood 3905.8 ± 296.6 pg/ml; p = 0.029).

The peripheral blood non-T cells were divided into CD19+ B cell group and CD14+ monocyte group using MACS separation methods. Both cell groups produced a small amount of MIF constitutively and with LPS stimulation. However, after the cells were stimulated with CpG, CD14+ cells produced much more MIF than CD19+ cells (Figure 3C).

*Stimulation of MIF production in RA synovial fibroblasts*. To determine the potent stimulators of MIF induction from RA synovial fibroblasts, we used IL-17 and CD40 ligand (CD40L) as T cell cytokines, and IL-1ß and TNF-α as proinflammatory cytokines. RA synovial fibroblasts were cultured with 10 ng/ml of IL-17, CD40L, and IL-1ß, and 1 ng/ml of TNF-α for 48 h, and the MIF concentrations in the conditioned media were measured by sandwich ELISA. The production of MIF was increased in the synovial fibroblasts treated with IL-1ß (111.8 ± 7.8 pg/ml vs 52.5 ± 3.6 pg/ml untreated; $p < 0.001$) and IL-17 (111.6 ± 8.3 pg/ml; $p < 0.001$) alone, but not in those treated with TNF-α or CD40L alone. However, after RA synovial fibroblasts were stimulated by various kinds of costimulation of the cytokines, the production of MIF was significantly augmented (Figure 4).

*Correlation of serum and SF levels of MIF and VEGF*. To determine the relationship of MIF with VEGF, we measured serum and SF levels of VEGF in the same patients with RA and OA, and serum VEGF levels in the healthy volunteers by sandwich ELISA. The serum VEGF level was higher in patients with RA than in either patients with OA (0.92 ± 0.06 ng/ml vs 0.54 ± 0.06 ng/ml; p = 0.001) or healthy volunteers (0.42 ± 0.08 ng/ml; $p < 0.001$), and it was also higher in the SF of patients with RA than in patients with OA (2.39 ± 0.2 ng/ml vs 1.2 ± 0.1 ng/ml; $p < 0.001$; Figures 5A, 5B). Both serum and SF levels of MIF were found to correlate well with VEGF levels ($r = 0.52$, $p < 0.001$ and $r = 0.6$, $p < 0.001$, respectively; Figures 5C, 5D). However, there was no correlation between MIF and VEGF levels in OA serum and SF (data not shown).

*rhMIF induces VEGF and IL-8 in RA synovial fibroblasts*. After synovial fibroblasts were stimulated with rhMIF, VEGF and IL-8 production was measured in the conditioned media. RA synovial fibroblasts were cultured in the presence of rhMIF (1–100 ng/ml), and the concentrations of VEGF and IL-8 were determined by sandwich ELISA. Low levels of constitutive VEGF and IL-8 were detectable in nonstimulated RA synovial fibroblasts. With the stimulation of rhMIF, the production of VEGF and IL-8 was enhanced in a dose-dependent manner. As shown in Figure 6, the production of VEGF and IL-8 was maximal when rhMIF was given in a concentration of 10 ng/ml, and gradually declined at higher doses. There was neither cytotoxic effect nor proliferative effect on RA synovial fibroblasts at the experimental concentrations of rhMIF (data not shown).

*rhMIF induces angiogenesis in vitro*. To confirm the effects of

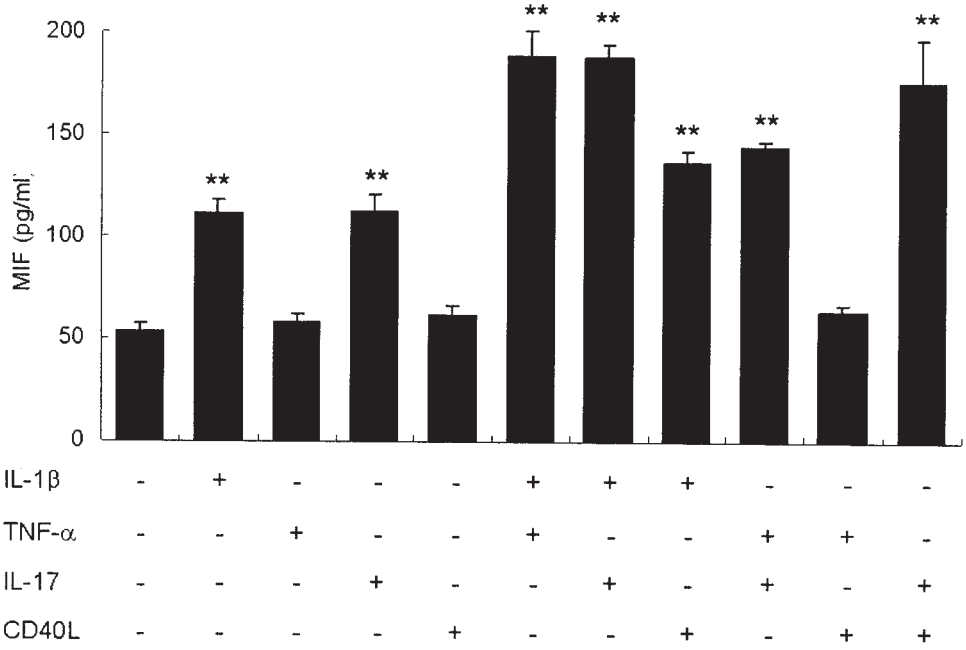



*Figure 4*. Additive effects of cytokines on MIF production in RA synovial fibroblasts stimulated with TNF-α (1 ng/ml), IL-1ß (10 ng/ml), IL-17 (10 ng/ml), and CD40 ligand (CD40L, 10 ng/ml) for 48 h. Levels of MIF were measured in conditioned media by sandwich ELISA. Bars show means and SEM of 6 separate experiments. **p < 0.005 compared with MIF production of unstimulated cells.

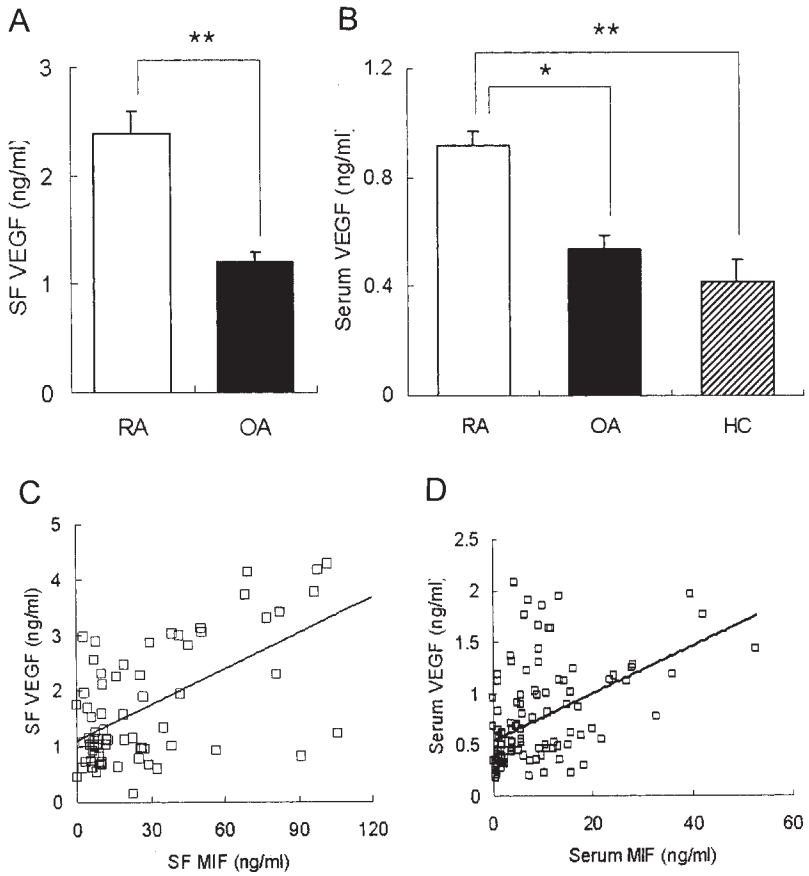



*Figure 5*. A. SF levels of vascular endothelial growth factor (VEGF) in patients with RA and OA; B. Serum levels of VEGF in patients with RA and OA and controls (HC). Both serum and SF levels of VEGF were significantly higher in patients with RA than the controls. *p < 0.05, **p < 0.001. C. Relationship between SF MIF and VEGF levels in patients with RA. D. Relationship between serum levels of MIF and VEGF in patients with RA. Both serum and SF levels of MIF were positively correlated with VEGF levels (r = 0.6, p < 0.001 and r = 0.52, p < 0.001, respectively).

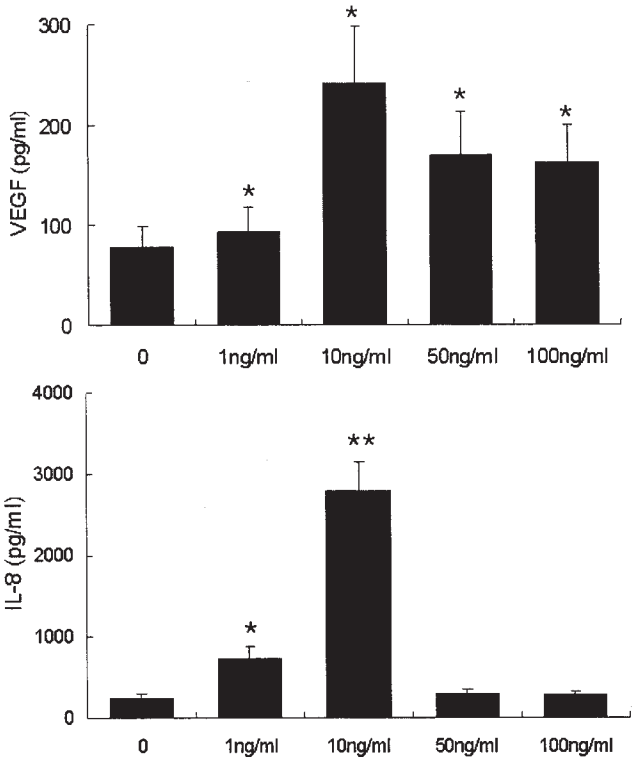



*Figure 6*. MIF induced production of VEGF and IL-8 by RA synovial fibroblasts. RA synovial fibroblasts were treated with rhMIF for 48 h, then concentrations of VEGF and IL-8 in conditioned media were measured by sandwich ELISA. Values represent means and SEM of 4 experiments. *$p < 0.05$, **$p < 0.005$ compared with the production of VEGF or IL-8 of unstimulated cells (0).

MIF on angiogenesis, we first studied tube-like structure formation, which is an *in vitro* angiogenesis in HUVEC stimulated with rhMIF. rhMIF, as well as rhVEGF (5 ng/ml), enhanced endothelial tube formation with maximal effect of 10 ng/ml (Figure 7A).

To determine the potential of MIF in angiogenesis of patients with RA, RA SFMC were treated with rhMIF, and then HUVEC were cultured with the conditioned media from rhMIF-pretreated SFMC. Tube formation was augmented in the HUVEC, which were incubated with the media from rhMIF-treated SFMC (tube length 11.5 ± 0.2 mm vs 20.1 ± 1.6 mm; $p = 0.01$), and this reaction was inhibited by anti-VEGF-neutralizing antibodies (tube length 20.1 ± 1.6 mm vs 13.5 ± 0.6 mm; $p = 0.01$; Figures 7B, 7C).

## DISCUSSION

RA is a chronic inflammatory joint disease, and complex networks incorporating many cytokines and chemokines are known to be involved in its pathogenesis. Thus, to understand the pathogenesis of RA, various cytokines should be considered together. MIF is one of the cytokines involved in the pathogenesis of RA[1-3,7]. It is also known to have a proinflammatory role in various diseases, such as sepsis[25], delayed-type hypersensitivity[26], glomerulonephritis[27], and malignancies[18-20]. Serum MIF levels are elevated in other autoimmune diseases, such as systemic lupus erythematosus (SLE)[28], psoriasis[29], Wegener's granulomatosis, and relapsing polychondritis[30]. Cerebrospinal fluid MIF levels are increased in neuro-Behçet's disease and in multiple sclerosis[31].

In the pathogenesis of RA, MIF is known to regulate the synovial hyperplasia by acting both directly and via the involvement of TNF-α and IL-1ß, to activate T cells[5,7]. MIF is released by T cells, macrophages, synovial fibroblasts, and endothelial cells in RA, and has both paracrine and autocrine effects with respect to the stimulation and activation of these cells[7]. MIF is also overexpressed in serum, SF, cultured synovial fibroblasts, and the synovial tissues of patients with RA[2,3,32]. One interesting study found that synovial MIF immunostaining is well correlated with RA disease activity as defined by CRP concentration[2]. Nevertheless, there are few data regarding quantitative MIF determinations and clinical correlations including CRP and ESR levels. In a recent study, it was shown that radiological joint damage correlates with circulating MIF levels and with high expression of MIF polymorphisms[33]. In this study, elevated SF levels of MIF, not serum MIF, were found to have strong correlation with clinical measures of disease activity and severity, e.g., ESR, CRP, and the presence of bony erosions, which was proven by sandwich ELISA. This finding indicates that synovial macrophages, fibroblasts, and T cells are activated to produce MIF in the more active and severe disease states. SF levels of MIF were much higher than serum levels, and SF levels of MIF were correlated with the clinical findings, indicating that the local tissue MIF response appears to be more marked than the systemic response. This observation is in accord with previous studies[1]. Also, because SF MIF levels were higher in patients with bony erosion than in those without, MIF may play a role in joint destruction via MMP and COX, as previous studies showed. While circulating MIF levels correlated with radiological joint damage in a recent study, synovial MIF levels, but not circulating MIF levels, had a correlation with bony erosions in our study. The distinction could be explained by the differences of disease activities, disease durations, and medication states of the subjects and the cross-sectional design limitation of our study. Although the radiologic scoring system or Disease Activity Score were not evaluated in this study, the relationship of MIF with ESR, CRP, steroid dosages, and the presence of bony erosions may suggest MIF could reflect disease activity and severity. But it remains to be seen whether the MIF levels change as a consequence of disease activity with therapies or if MIF is the cause of disease activity.

In RA, the main sources of MIF are infiltrating T cells in RA synovium[32], macrophages, and synovial fibroblasts[1]. We wanted to determine the main source of MIF in patients with RA *in vivo*, and we found both T cells and non-T cells from peripheral blood and SF release a similar degree of MIF constitutively. But with anti-CD3 stimulation, MIF production

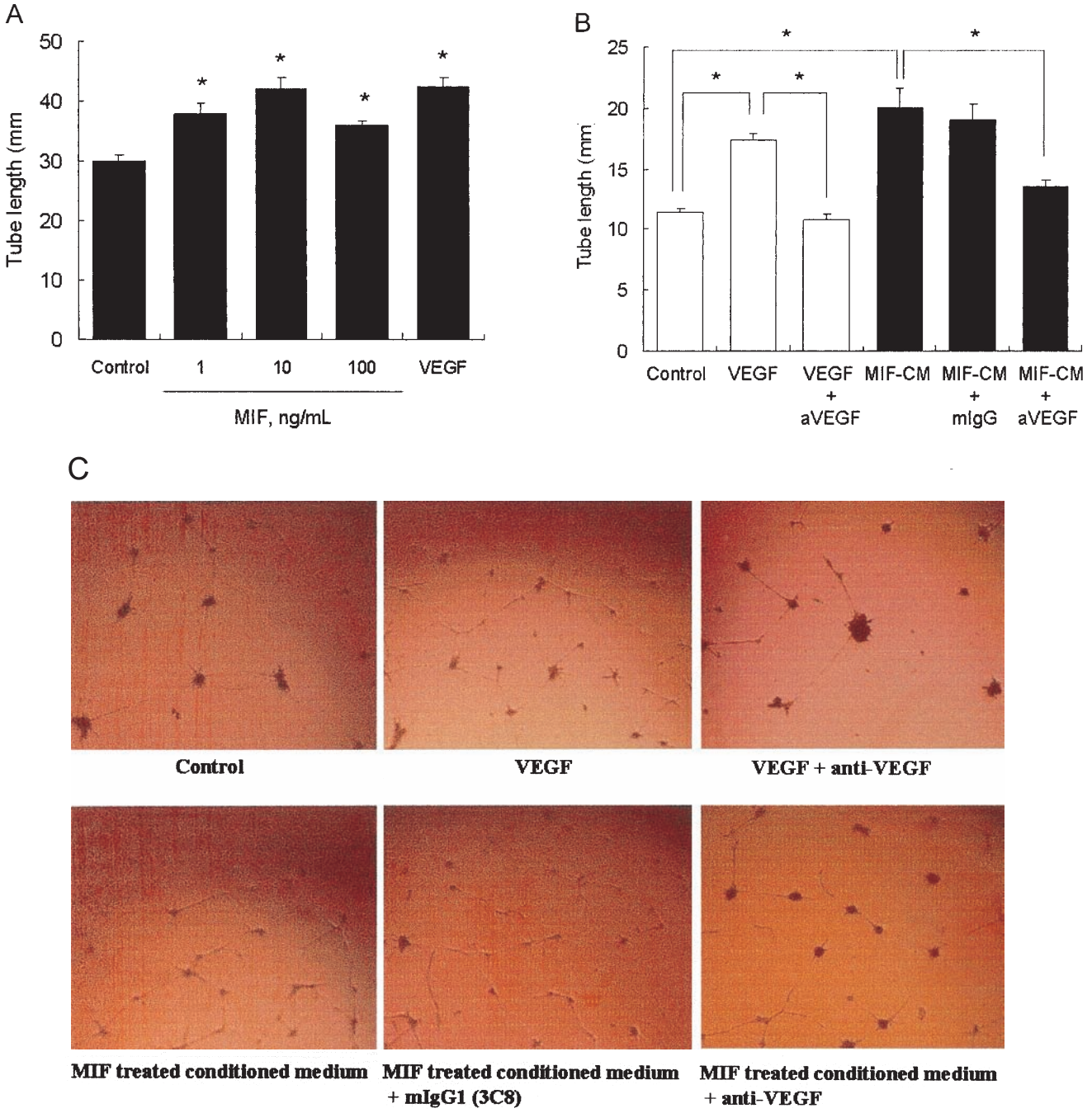



*Figure 7*. A. Human umbilical vein endothelial cells (HUVEC) were treated with rhMIF (1–100 ng/ml) and rhVEGF (5 ng/ml), and *in vitro* tube formation was observed. The MIF-induced endothelial tube formation was increased in a dose-dependent manner with a maximal concentration of MIF, 10 ng/ml. B, C. When HUVEC were cultured with the conditioned media from rhMIF-pretreated SFMC, tube formation was enhanced; however, enhancement was blocked by anti-VEGF antibodies. *$p < 0.05$ compared with the tube length (mm) of unstimulated cells (control). MIF-CM: conditioned media from MIF-pretreated RA SFMC.

was greatly enhanced in the activated T cells compared with LPS or CpG-stimulated non-T cells. This result suggests activated T cells are the main source of MIF in patients with RA. When the PBMC were divided into CD19+ B cell group and CD14+ monocyte group, both cell groups had low capacity for constitutive MIF production in a similar degree. But when cells were stimulated by CpG, the production of MIF was enhanced more in monocytes than in B cells. From this result, monocytes may be another main source of MIF in the chronic inflammatory disease of RA. Further, when the activated T cells were cocultured with non-T cells, the production of MIF was much augmented. This synergistic effect may be due to a

reciprocal action of the various cells, producing other inflammatory and proinflammatory cytokines. But the role of T cells and other cells in MIF production needs to be further investigated.

The angiogenesis is another characteristic finding of the inflammatory synovium in RA, and it has an important role in the destructive processes in cartilage and bone. VEGF is an important mediator of angiogenesis in the malignant and inflammatory diseases. We previously reported that serum VEGF levels are correlated with disease activity variables such as ESR, CRP, the titers of serum rheumatoid factor, and numbers of tender and swollen joints in patients with RA[17], and transforming growth factor-ß induces the production of VEGF in RA fibroblast-like synoviocytes[34]. MIF expression correlates with the VEGF expression in human glioblastomas[20], and it is upregulated during hypoxic stress in glial tumor[35]. MIF enhances the VEGF production in hepatocellular carcinoma and esophageal squamous cell carcinoma, and it may stimulate angiogenesis in hepatocellular carcinoma[11,18]. rhMIF also induces angiogenesis in the rat cornea *in vivo*[14]. We investigated whether MIF had a relationship with VEGF in patients with RA, like some malignant tumors, using 3 methods. First, we confirmed that serum and SF levels of MIF were well correlated with the VEGF levels in patients with RA, but not in those with OA. Next, we suspected MIF could have a potential role in modulating the angiogenesis by induction of the angiogenic factors, such as VEGF or IL-8, in RA synovial fibroblasts. To confirm this, we treated RA synovial fibroblasts with rhMIF and determined the production of VEGF and IL-8 by the cells. rhMIF increased the production of VEGF and IL-8 in the cultured synovial fibroblasts with an optimal dosage of 10 ng/ml. MIF is known to induce fibroblast-like synoviocyte proliferation[10], but we found no significant proliferation of synovial fibroblasts with our experimental concentrations of MIF. This suggests that the influence of MIF on the induction of VEGF and IL-8 is not mediated by cell proliferation. Finally, we tested whether MIF could induce the angiogenesis *in vitro*. After HUVEC were cultured with the conditioned media from rhMIF-treated RA SFMC, the endothelical tube formation was enhanced. This enhanced tube formation was prevented with anti-VEGF antibodies. These results suggest that MIF may indirectly participate in RA angiogenesis mediated by VEGF. Neoangiogenesis, which is typically observed in synovial hypertrophy and pannus formation, could be explained by such MIF-induced VEGF production by RA synovial fibroblasts and the vessel formation by endothelial cells. This is the first investigation showing that MIF can induce angiogenesis in RA. More detailed research including *in vivo* study is needed to confirm the additional roles of MIF in RA angiogenesis. Proceeding from these results, MIF may have a central role in the aggravation of tissue inflammation, by stimulation of angiogenesis, as well as by induction of inflammatory cytokines. MIF induced the proliferation of RA synovial fibroblasts. Then high metabolic demands of inflamed synovial tissues and the rapid rate of synovial proliferation induce the hypoxic state in RA[35], and this hypoxic stress may stimulate MIF expression again, like glial tumor[36], resulting in the induction of angiogenesis, as well as the deterioration of inflammation. These reciprocal actions of hypoxia, MIF, and angiogenesis may result in aggravation of RA synovial lesions.

It is known that endogenous glucocorticoids participate in the regulation of inflammatory processes and that MIF plays a role in the counterregulation of glucocorticoids[1,5]. Glucocorticoids have a biphasic regulatory effect in the expression of MIF in RA synoviocytes. For example, low concentration of dexamethasone induces MIF production; on the other hand, high concentration of dexamethasone suppresses MIF expression[1]. According to our observation, the daily dosage of oral prednisolone is significantly correlated with ESR, CRP, and SF MIF levels, and patients taking prednisolone $\geq$ 5 mg/day had higher MIF levels than patients taking prednisolone $<$ 5 mg/day. This is consistent with the finding that serum MIF is positively associated with current corticosteroid dose in SLE[28]. However, viewing this issue from another standpoint, RA patients with more severe and more active disease generally need more steroids in our clinical setting, and thus the correlation between SF MIF and steroid dose may reflect a correlation between SF MIF levels and disease activity and severity. This is supported by evidence that current dosage of oral prednisolone correlates with the indicators of disease activity such as ESR and CRP.

Our results indicate that MIF is overexpressed in sera and in SF in RA, and that it is correlated with the clinical measures of RA. MIF levels correlate with VEGF levels in sera and SF of patients with RA. rhMIF stimulates VEGF and IL-8 production in RA synovial fibroblasts and induces endothelial tube formation *in vitro*. These results suggest that MIF may be an important cytokine in modulation of angiogenesis as well as in the perpetuation of the inflammatory processes in RA. Moreover, it becomes evident that MIF represents a potential therapeutic target in the treatment of RA.

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