

Midkine, a Heparin-Binding Growth Factor, Is Fundamentally Involved in the Pathogenesis of Rheumatoid Arthritis

Kiyoko Maruyama, Hisako Muramatsu, Naoki Ishiguro, and Takashi Muramatsu

Objective. Midkine (MK), a heparin-binding growth factor, promotes growth, survival, and migration of various cells. The essential role of MK in migration of inflammatory cells has been shown using mice deficient in the MK gene ($Mdk^{-/-}$ mice). We undertook this study to investigate the role of MK in the pathogenesis of rheumatoid arthritis (RA).

Methods. MK levels in specimens from patients were determined by enzyme-linked immunosorbent assay, and localization of MK was revealed by immunohistochemical analysis. Susceptibility to antibody-induced arthritis was compared between $Mdk^{-/-}$ and wild-type (WT) mice. Osteoclast differentiation was monitored using macrophage-like cells isolated from human synovial tissue and macrophages from mouse bone marrow.

Results. MK levels in sera and synovial fluid were increased in most RA patients, indicating a strong correlation between MK expression and RA. MK was expressed in macrophage-like cells and fibroblast-like cells in synovial membranes from the patients. In antibody-induced arthritis, $Mdk^{-/-}$ mice seldom developed the disease, while most of the WT mice did. Administration of MK to the $Mdk^{-/-}$ mice increased the frequency of antibody-induced arthritis. Migration of inflammatory leukocytes to the synovial membranes in the disease model was suppressed in the $Mdk^{-/-}$

mice. Furthermore, MK was found to promote the differentiation of osteoclasts from macrophages.

Conclusion. MK participates in each of the two distinct phases of RA development, namely, migration of inflammatory leukocytes and osteoclast differentiation, and is a key molecule in the pathogenesis of RA.

Rheumatoid arthritis (RA) is a destructive and progressive inflammatory disease of the joints, leading to joint deformation, which markedly diminishes the quality of life of patients. Various cytokines are involved in the pathogenesis of RA. Their primary roles are in lymphocyte activation, migration of inflammatory leukocytes, and osteoclast differentiation (1). Chemokines are involved in leukocyte migration, and receptor activator of NF- κ B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) are involved in osteoclast differentiation (2,3). Interleukin 6 (IL-6) mainly acts at the stage of lymphocyte activation (4,5). IL-1 and tumor necrosis factor α (TNF α) induce chemokine synthesis and activate synovial cells and osteoclasts (1,6,7). In addition, TNF α has been reported to induce the differentiation of osteoclasts by a different route from RANKL (8). IL-17 and IL-18 play roles similar to those of IL-1 and TNF α (9). Recently, monoclonal antibodies to TNF α and antagonists to TNF α receptor and IL-1 receptor have been introduced as therapeutic agents for RA and have yielded promising results (10,11), although side effects, particularly infections, have been encountered in certain cases (12,13).

It should be emphasized that we still do not have the entire picture of the cytokines which play roles in the development of RA. This understanding is important for the prevention, diagnosis, and treatment of RA. For example, some of the optimal targets of anticytokine therapy may still remain to be discovered.

Here, we report that a heparin-binding growth factor, midkine (MK) (14–16), is fundamentally involved in the pathogenesis of RA. This molecule regulates two

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critical steps of RA development, namely, the migration of inflammatory leukocytes and osteoclast differentiation. MK is a 13-kd protein (14) and has 45% sequence identity to pleiotrophin, also called HB-GAM (17–19). MK enhances growth, survival, and migration of various cells (14,19–24). It also promotes chondrocyte differentiation (25) and enhances fibrinolytic activity of vascular endothelial cells (26). The ability of MK to enhance the migration of neutrophils and macrophages correlates with its involvement in certain inflammatory diseases (22,23). We have already found that in mice deficient in the MK gene ($Mdk^{-/-}$ mice), neointima formation (22) and nephritis (23) after ischemic injury are less severe than in wild-type (WT) mice.

PATIENTS AND METHODS

Clinical specimens. Samples of synovial fluid and sera were obtained from patients with RA or osteoarthritis (OA). Synovial fluid was aspirated from the knee joints of RA and OA patients as part of their therapeutic program. All RA patients fulfilled the 1987 American College of Rheumatology (ACR; formerly, the American Rheumatism Association) revised classification criteria (27), and all OA patients met the ACR criteria for the classification of knee OA (28). All patients were being treated with nonsteroidal antiinflammatory drugs and disease-modifying antirheumatic drugs. The 145 RA patients had a mean age of 63.1 years (range 17–85 years), while the 18 OA patients had a mean age of 67.2 years (range 54–78 years). In most RA patients, the Larsen grade (29) and the ACR revised global functional class (30) were determined. The number of patients in each Larsen grade was as follows: grade 1, 9; grade 2, 24; grade 3, 27; grade 4, 39; and grade 5, 46. The number of patients in each ACR functional class was as follows: class I, 22; class II, 34; class III, 41; and class IV, 48.

The MK concentration was determined as described previously (31). Briefly, affinity-purified rabbit anti-human MK antibody (31) was adsorbed to the well of a microtiter plate, and then a sample solution containing MK was added to the well. Biotinylated anti-human MK antibody was then added, and the absorbed biotin-labeled antibody was quantified enzymatically with the aid of avidin-labeled β -galactosidase and its fluorogenic substrate. Rheumatoid factor (RF) in the sera of RA patients was measured using a reagent from Eiken Kagaku (Tokyo, Japan) according to the manufacturer's instructions. All patients who provided specimens (including surgical specimens) completed and signed an informed consent document that had been approved by the Nagoya University Review Board.

Animals. The generation of $Mdk^{-/-}$ mice has been described previously (32). Heterozygous mutant mice ($Mdk^{+/-}$ mice) were backcrossed to C57BL/6 mice 7 or 8 times and then mated with each other. The WT or $Mdk^{-/-}$ mice obtained in this way were inbred once, and the male offspring were used as WT or $Mdk^{-/-}$ mice for experiments. The ages of the WT and $Mdk^{-/-}$ mice did not differ by >1 week in each experiment. The ddY-strain mice were purchased from Japan SLC (Shizuoka, Japan).

All mice used in these experiments were 6–8 weeks old and were bred and maintained with sterilized food, water, and bedding at the Animal Facility of the Nagoya University School of Medicine. All experiments were approved by the Animal Ethics Committee of Nagoya University.

Mouse arthritis models. Each mouse received 8 mg of arthritogenic monoclonal antibody cocktail (Iwai Chemicals, Tokyo, Japan), which contains anti-type II collagen antibodies, as previously described (33). Intraperitoneal (IP) injections were performed on 2 consecutive days. On day 4, 100 μ l of 500 μ g/ml lipopolysaccharide (LPS) in phosphate buffered saline (PBS) was injected IP. The severity of arthritis was judged on a scale of 0–4 for each limb (0 = normal, 1 = mild but definite redness and swelling of the ankle or wrist, 2 = moderate-to-severe redness and swelling of the ankle or wrist, 3 = redness and swelling of the entire foot, and 4 = maximally inflamed limb with involvement of multiple joints) (33). Values obtained for the 4 limbs were added. In the pump study, human MK in saline (1 mg/ml) or human serum albumin (Wako, Osaka, Japan) in saline (1 mg/ml) was infused into $Mdk^{-/-}$ mice using an osmotic pump (Alza, Palo Alto, CA). The pumps, which were implanted under the abdominal skin, infused a total of 90 μ l continuously over a period of 7 days. Human MK was produced in yeast (34) and was kindly provided by Dr. S. Sakuma at Cell Signals (Yokohama, Japan). Levels of MK (31) and TNF α were measured in the sera of all mice. The assay reagents for TNF α were purchased from BioSource International (Camarillo, CA).

Histology and immunohistochemistry. Mouse wrists were excised, fixed for 7 days in 10% formalin solution (Wako), and decalcified with 0.5 moles/liter of EDTA (35). After dehydration, the tissues were embedded in paraffin and cut into sections 3- μ m thick through the long axis. Serial sections were mounted on slides, dried overnight, and stored in an airtight box. Sections were stained with hematoxylin and eosin. Sections were also treated with 0.3% H₂O₂ in PBS for 30 minutes at room temperature, blocked with 1% bovine serum albumin (BSA) in PBS for 20 minutes, and stained with rabbit anti-mouse MK antibody, rat anti-mouse F4/80 (macrophage) antibody (Serotec, Raleigh, NC), or rat anti-mouse neutrophil antibody (Serotec) as the first antibody at 4°C overnight, followed by horseradish peroxidase (HRP)-labeled affinity-purified goat anti-rabbit IgG or HRP-labeled affinity-purified goat anti-rat IgG (Jackson ImmunoResearch, West Grove, PA) as the second antibody. The staining was visualized by diaminobenzidine tetrahydrochloride (Amersham Pharmacia Biotech, Tokyo, Japan). The rabbit anti-mouse MK antibody was prepared as described previously (20).

Synovial tissue was surgically removed from 60–65-year-old female patients with RA (ACR functional class IV, Larsen grade 5) who had not received steroids before surgery. The synovial tissue was immediately cooled on ice and processed as described above to prepare sections, except that decalcification was not performed. Sections were treated with 0.1% Triton X-100 in PBS for 15 minutes at room temperature, blocked with 3% BSA in PBS for 30 minutes, and then stained with rabbit anti-human MK antibody (31) and/or mouse monoclonal anti-CD68 (macrophage marker) antibody (Neomarker, Fremont, CA) at 4°C overnight, followed by signal amplification with Alexa Fluor 594 goat anti-rabbit IgG and/or Alexa Fluor 488 goat anti-mouse IgG (Molecular

Probes, Eugene, OR) for 1 hour at room temperature. Specimens were then examined using a fluorescence microscope (Axioskop 2 plus; Zeiss, Wetzlar, Germany).

For staining of the cells from synovial membranes, cells were fixed with 4% paraformaldehyde at room temperature for 20 minutes, washed with PBS, treated with 0.1% Triton X-100 in PBS for 9 minutes, washed with PBS, and treated with 1% normal goat serum for 20 minutes. The cells were then reacted with rabbit anti-human MK antibody. Fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Sigma, St. Louis, MO) was used as the second antibody, and specimens were observed using a confocal laser scanning imaging system (MRC1024; Nippon Bio-Rad, Tokyo, Japan).

Separation of macrophage-like cells and fibroblast-like cells. Suspended cells were isolated from synovial tissues according to the method previously described (36). CD14+ cells were separated from the suspended cells by the magnetic-activated cell sorting system using anti-human CD14 monoclonal antibody-coated beads and a column (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The CD14+ cells were identified as macrophage-like cells by staining with anti-CD68 and by their morphology. Cells that passed through the column of CD14-coated beads were cultured and passaged using 0.125% trypsin and 0.01% EDTA on a 2-well chamber slide (Nalge Nunc International, Naperville, IL). The adherent cells were regarded as fibroblast-like cells based on their morphology. The medium used for culture of separated cells was Dulbecco's modified Eagle's minimum essential medium with 10% fetal calf serum (FCS).

Osteoclast differentiation. Isolated CD14+ cells (10^6 /well) were seeded on a 2-well chamber slide and cultured in the presence (100 ng/ml) or absence of recombinant human M-CSF (Chemicon, Temecula, CA) and/or RANKL (50 ng/ml; PeproTech EC, London, UK) and/or human MK in Dulbecco's modified Eagle's minimum essential medium with 10% FCS for 3 weeks. Medium was changed every 3 days.

M-CSF-dependent bone marrow macrophages were prepared from whole bone marrow of male ddY mice and cultured for 2 days with recombinant mouse M-CSF (10 ng/ml; R&D Systems, Minneapolis, MN), which primes these cells to undergo osteoclast differentiation (37,38). The cells were then cultured in the presence or absence of the following factors for 3 days: mouse M-CSF (10–50 ng/ml), RANKL (100 ng/ml), and human MK. Both human and mouse osteoclasts were identified by tartrate-resistant acid phosphatase (TRAP) staining (35). TRAP-positive cells were counted in the entire area of a well. In each experiment, 4 wells were counted and the average value is shown. The result was reproducible in 3 independent experiments.

Statistical analysis. After correlation analysis of human samples, statistical analysis was performed using Fisher's exact test. *P* values less than 0.05 were considered significant. For the animal model and culture cells, statistical comparisons were performed by Student's *t*-test.

RESULTS

Increased MK levels in specimens from RA patients. At first, we used samples from patients to investigate the correlation between MK expression and RA.

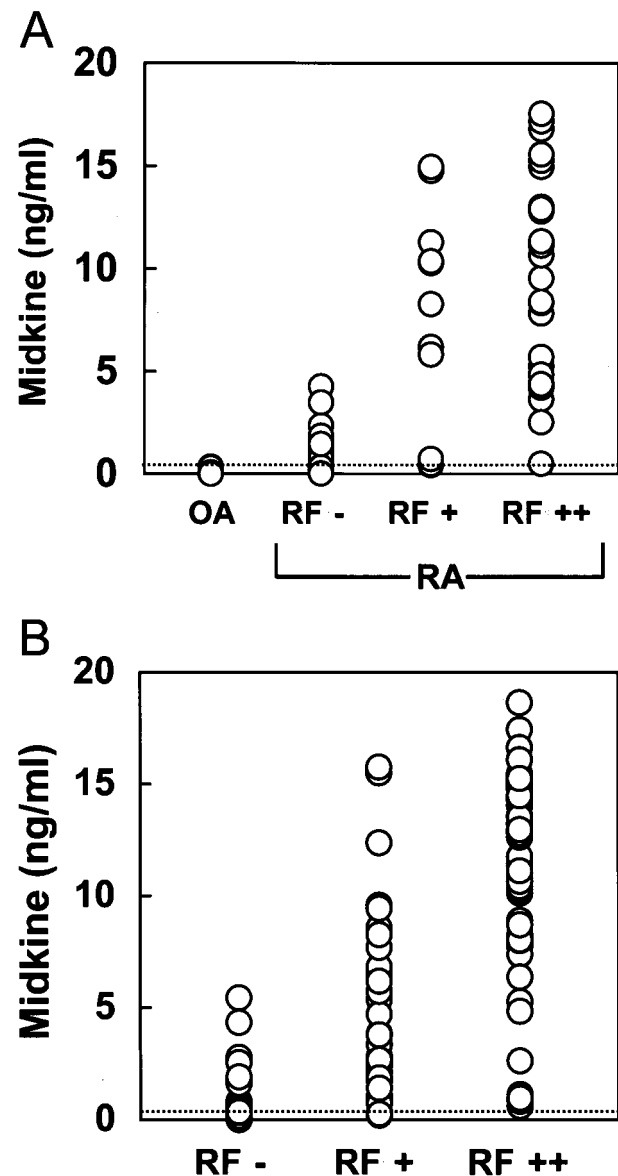


Figure 1. Increased levels of midkine (MK) in specimens from patients with rheumatoid arthritis (RA). **A**, Enzyme-linked immunosorbent assay (ELISA) of MK in synovial fluid from patients with RA ($n = 55$) or osteoarthritis (OA) ($n = 18$). Dotted line shows the cutoff level (332 pg/ml), which corresponds to the highest MK value in OA patients. RA patients were classified as strongly positive for rheumatoid factor (RF++) ($n = 26$), moderately positive for RF (RF+) ($n = 11$), and negative for RF (RF-) ($n = 18$). **B**, ELISA of MK in the sera of RA patients. Dotted line shows the cutoff level (600 pg/ml), which was set in previous studies (see refs. 31 and 34). There were 51 RF++ patients, 37 RF+ patients, and 32 RF- patients.

The MK levels were determined by enzyme-linked immunosorbent assay (ELISA) of the synovial fluid and sera. In the majority of cases, a high MK value was

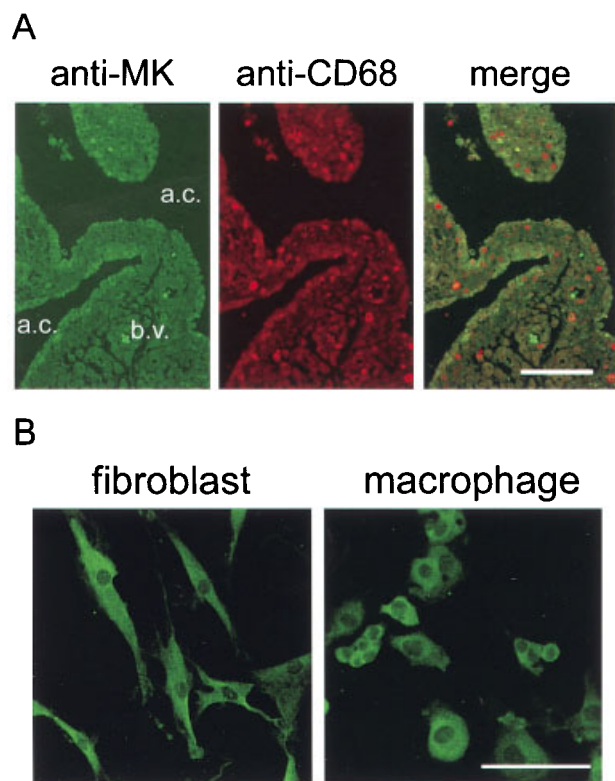


Figure 2. MK expression in inflamed synovial membranes from RA patients. **A**, Double staining of tissue sections by antimacrophage (anti-CD68) and anti-MK antibody. Erythrocyte staining by anti-MK antibody was observed in control staining (without anti-MK antibody or absorbed anti-MK antibody) and was nonspecific. Synovial membrane was obtained from a female patient (age 62 years, American College of Rheumatology functional class IV, Larsen grade 5). **a.c.** = articular cavity; **b.v.** = blood vessel. Bar = 200 μm . **B**, Fibroblast-like or macrophage-like cells from synovial membranes were grown on a 2-well chamber slide and stained by anti-MK antibody. Bar = 100 μm . See Figure 1 for definitions.

observed in the synovial fluid from RA patients (Figure 1A), while only low levels of MK were found in that from OA patients. Taking the highest MK value determined in OA patients (332 pg/ml) as the cutoff level, 85.4% of RA patients had higher values. The MK levels correlated with the serum levels of RF in RA patients ($r = 0.683, P < 0.001$). When the patients were classified into those with a high level of RF (RF++), a medium level of RF (RF+), and no RF (RF-), all RF+ or RF++ patients exhibited a value greater than the cutoff value. Even 10 of 18 RF- patients exhibited a higher value. The average MK values for RF++, RF+, and RF- patients were 9,670 pg/ml, 7,590 pg/ml, and 1,240 pg/ml, respectively.

The serum MK value was also elevated in RA patients. In normal human subjects, the serum MK value did not reach 600 pg/ml (31,34). Taking this as a cutoff value, 91.6% of RA patients had elevated values (Figure 1B). Serum MK values also correlated with RF levels ($r = 0.744, P < 0.001$), but not with the C-reactive protein level, erythrocyte sedimentation rate, ACR functional class, Larsen grade, age, or duration of disease. All RF++ patients had elevated values, as did 94.6% of RF+ patients (Figure 1B). Even 71.9% of RF- patients had elevated values (Figure 1B). The average MK values for RF++, RF+, and RF- patients were 9,790 pg/ml, 5,240 pg/ml, and 854 pg/ml, respectively. The serum MK level correlated well with the MK level in synovial fluid from the same patient ($r = 0.623, P = 0.002$). The possibility that anti-MK antibody cross-reacted with RF was excluded, since upon gel filtration of the sera, MK immunoreactivity was eluted at low molecular weight fractions (data not shown).

We investigated the origin of the elevated MK. Synovial membranes from RA patients are mainly composed of macrophage-like cells and fibroblast-like cells. Anti-MK antibody strongly stained cells facing the articular cavity, and the cells were also stained by anti-CD68 (a macrophage marker) (Figure 2A). Other cells in the synovial membrane were moderately stained by anti-MK antibodies. We stained samples from 4 patients, all of whom were at ACR functional class IV and Larsen grade 5, and obtained essentially identical results. Concordant with results of the immunohistochemical staining, both macrophage-like cells and fibroblast-like cells isolated from the synovial membrane expressed MK (Figure 2B).

Table 1. Differences in the frequency of antibody-induced arthritis between wild-type (WT) mice and mice deficient in the midkine (MK) gene (Mdk^{-/-} mice)*

Mice	No. of mice		Incidence, %
	With arthritis	Without arthritis	
WT	6	1	85.7†
Mdk ^{-/-}			
No treatment	1	9	10.0
HSA pump	3	8	27.3
MK pump	9	3	75.0

* Mice were evaluated daily for the presence of arthritis and judged to be arthritic if ≥ 1 joint (wrist, ankle) was red and swollen. The supply of MK to the peritoneum of Mdk^{-/-} mice by osmotic pump (MK pump) significantly increased the frequency of arthritis development compared with the supply of human serum albumin by osmotic pump (HSA pump) ($P < 0.05$).

† $P < 0.05$ versus untreated Mdk^{-/-} mice.

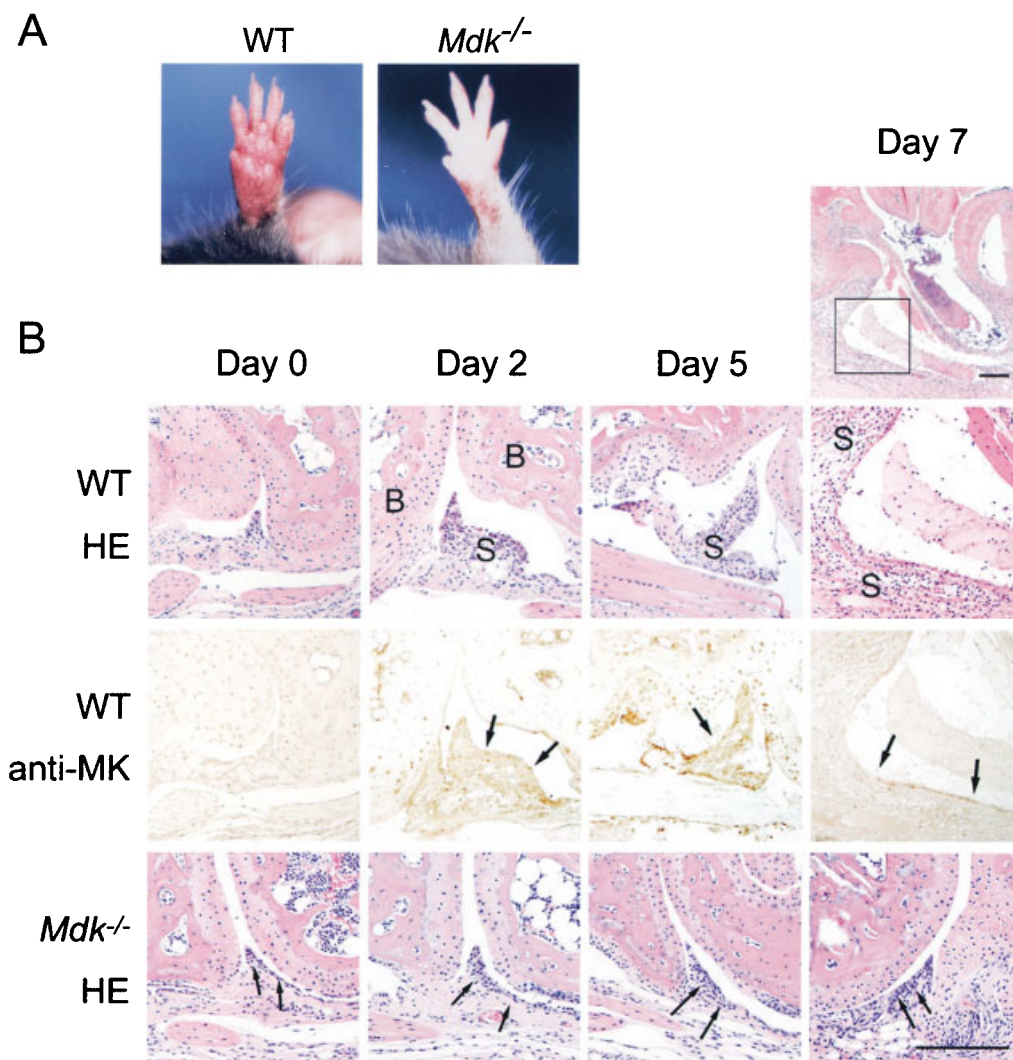


Figure 3. Suppression of antibody-induced arthritis in mice deficient in the midkine (MK) gene (*Mdk*^{-/-} mice). **A**, Representative photographs of paws of wild-type (WT) or *Mdk*^{-/-} mice 7 days after injection of anti-type II collagen antibodies. **B**, Sections of wrists stained by hematoxylin and eosin (HE) or anti-MK antibodies on day 0 and on days 2, 5, and 7 after antibody injection. Synovial membranes in *Mdk*^{-/-} mice were much thinner than those in WT mice on days 5 and 7 (arrows). MK expression was observed in synovial cells (S) of WT mice, particularly in the lining cells of the joint space (arrows). Arrows also show synovial cells in *Mdk*^{-/-} mice. The boxed area under day 7 was enlarged in the two photographs shown directly below. Bars = 200 μ m. B = bone.

Suppression of antibody-induced arthritis in *Mdk*^{-/-} mice. The high MK expression in specimens from RA patients appeared to indicate a relationship between MK expression and the pathogenesis of RA, and this prompted us to investigate this possibility further. A typical experimental model of RA is an antibody-induced arthritis, which is induced by injection

of anti-type II collagen antibody followed by injection of LPS (33). We investigated whether antibody-induced arthritis occurs in *Mdk*^{-/-} mice.

Rapid swelling of the joint occurred in WT mice on days 5 and 6 after antibody administration, and most mice developed evident arthritis on day 7 (Table 1 and Figure 3A). However, in most *Mdk*^{-/-} mice, arthritis

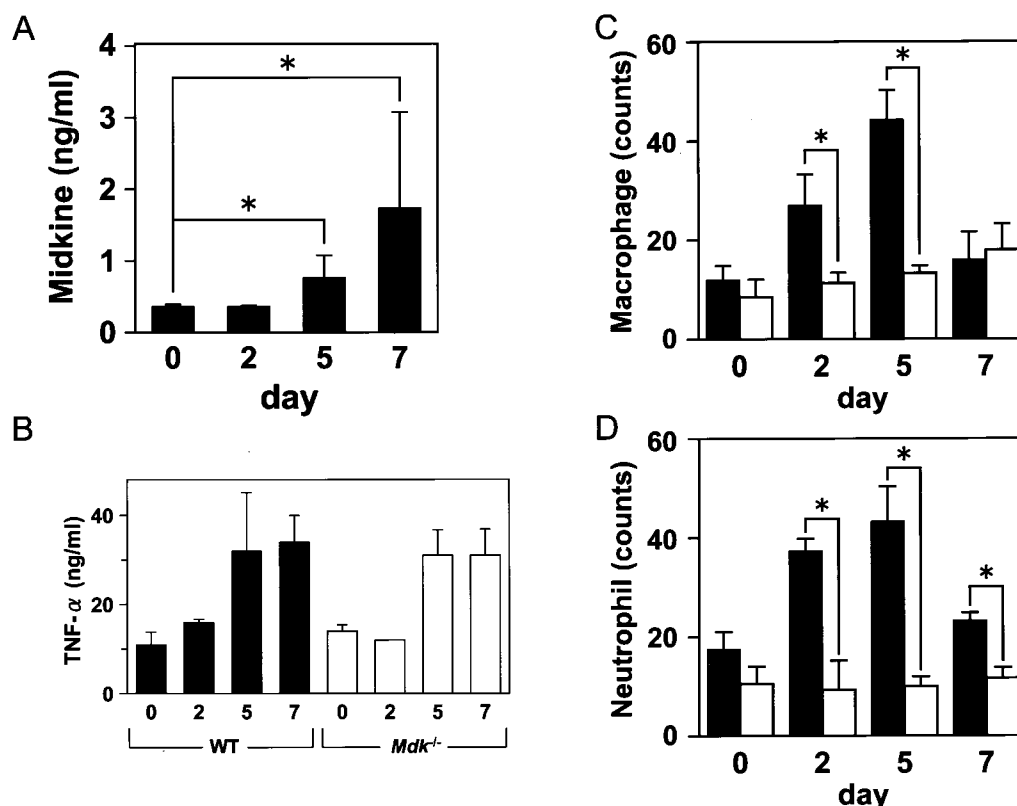


Figure 4. Serum levels of cytokines and numbers of infiltrated leukocytes on day 0 and on days 2, 5, and 7 of antibody-induced arthritis. **A**, Serum levels of MK in WT mice. **B**, Serum levels of tumor necrosis factor α (TNF α) in WT mice (solid bars) or Mdk^{-/-} mice (open bars). **C**, Numbers of macrophages in synovial membranes of WT or Mdk^{-/-} mice. **D**, Numbers of neutrophils in synovial membranes of WT or Mdk^{-/-} mice. Numbers of neutrophils and macrophages were counted in 3 randomly selected fields under 400 \times magnification, and average values are shown. * = $P < 0.05$. See Figure 3 for other definitions.

was not found on day 7 (Table 1 and Figure 3A) or later. The average arthritis score was 8.3 for WT mice and 0.7 for Mdk^{-/-} mice on day 7. Histologic examination revealed a proliferation of synovial cells on days 5 and 7 (Figure 3B) and an increase in joint cavity size and accumulation of synovial fluid on day 7 in WT mice (Figure 3B). Synovial membranes in Mdk^{-/-} mice were much thinner than those in WT mice on days 5 and 7 (Figure 3B). The increase in joint cavity size was not observed in Mdk^{-/-} mice on day 7 (Figure 3B). By immunohistochemical staining, cells in the synovial membrane of WT mice became more strongly stained by anti-MK on day 2 compared with day 0 (Figure 3B). The staining increased further on days 5 and 7, especially on cells facing the joint cavity. ELISA of the sera confirmed that the MK content increased progressively during arthritis pathogenesis (Figure 4A).

For the next step of analysis, we wished to

establish that the difference in arthritis development between Mdk^{-/-} and WT mice was actually due to the presence or absence of Mdk. WT mice are C57BL/6 mice, and Mdk^{-/-} mice were backcrossed 7 or 8 times to C57BL/6 mice, and the original strain consisted of 129Sv mice. One might argue that a small amount of gene derived from 129Sv mice made Mdk^{-/-} mice resistant to arthritis induction. However, this possibility is unlikely because C57BL/6 mice are relatively resistant to arthritis induction and require a high dose of antibody to induce arthritis. We confirmed that under the identical conditions, all of 5 129Sv mice developed arthritis as in C57BL/6 mice. Furthermore, the supply of MK to the peritoneum of Mdk^{-/-} mice by osmotic pump increased the frequency of arthritis development compared with the supply of human serum albumin (Table 1). This experiment definitively established that MK is a key factor in development of the arthritis.

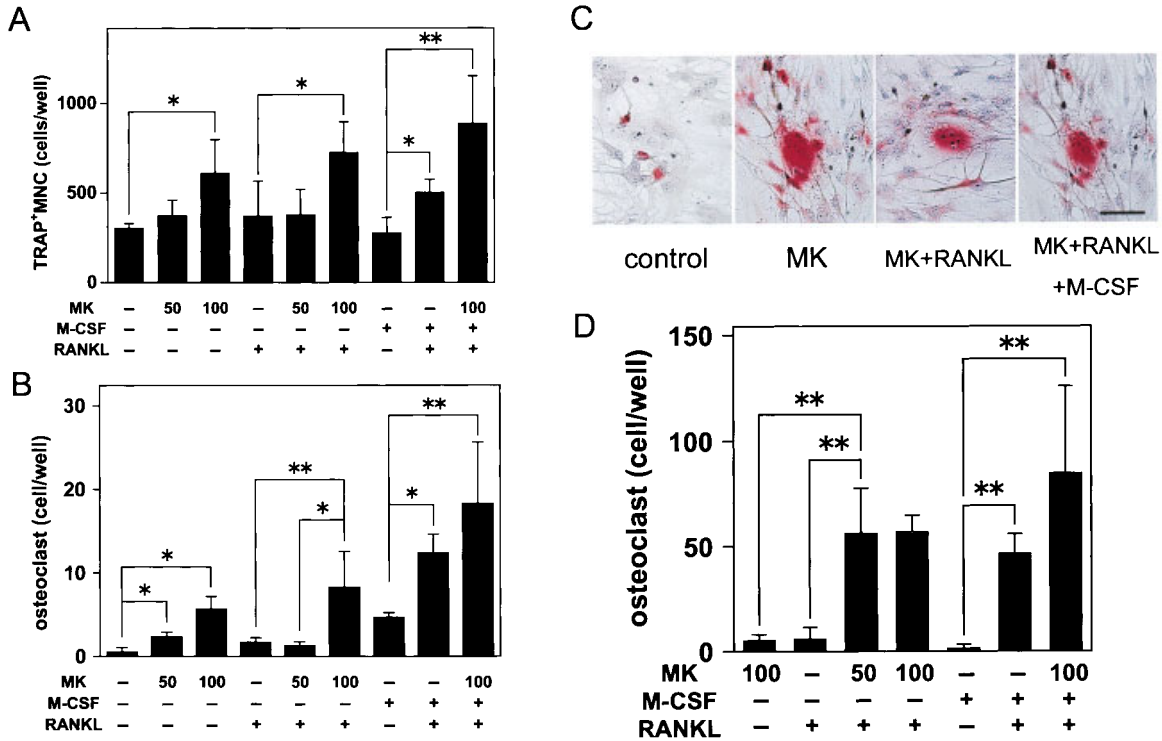


Figure 5. Promotion of osteoclast differentiation from macrophages by MK. **A**, Macrophage-like cells from the synovial membranes of a female RA patient (age 62 years, American College of Rheumatology functional class IV, Larsen grade 5) were cultured with factors (10–50 ng/ml mouse macrophage colony-stimulating factor [M-CSF], 100 ng/ml of receptor activator of NF- κ B ligand [RANKL], and human MK [concentrations shown in ng/ml]) and stained with tartrate-resistant acid phosphatase (TRAP). MK enhanced the differentiation of TRAP-positive multinuclear cells (TRAP⁺MNC) from the human macrophage-like cells alone, in combination with RANKL, or in combination with M-CSF and RANKL. **B**, Differentiation of osteoclasts from the human macrophage-like cells was enhanced by MK alone, MK in combination with RANKL, or MK in combination with RANKL and M-CSF. Multinuclear TRAP-positive cells were regarded as osteoclasts. **C**, Representative photographs of TRAP-positive cells differentiated from macrophage-like cells of the synovial membranes by culturing with the above factors. Bar = 50 μ m. **D**, Osteoclast differentiation from mouse macrophages by RANKL or RANKL and M-CSF was enhanced by MK. * = $P < 0.05$; ** = $P < 0.01$. See Figure 1 for other definitions.

Enhancement of both inflammatory cell migration and osteoclast differentiation by MK. We investigated the role of MK in the pathogenesis of antibody-induced arthritis. The serum level of TNF α did not differ between WT and Mdk^{-/-} mice (Figure 4B). Therefore, MK is involved in arthritis pathogenesis in ways that differ from those of TNF α . We found that fewer macrophages and neutrophils migrated to the synovial membrane in Mdk^{-/-} mice than in WT mice (Figures 4C and D). Thus, decreased migration of inflammatory leukocytes in Mdk^{-/-} mice is one of the reasons for the decrease in arthritis in these mice, as in the case of neointima formation (22) and nephritis (23) after ischemia. Since osteoclasts play central roles in bone destruction (39), we investigated whether MK promotes the differentiation of osteoclasts. So far, M-CSF and

RANKL, in combination but not alone, are known to induce the differentiation of osteoclasts from macrophages (2,37). We cultured macrophage-like cells from the synovial membranes of RA patients with MK or other cytokines. Osteoclast differentiation was evaluated by staining for TRAP, which is expressed in these cells. MK at 100 ng/ml significantly increased the number of TRAP-positive cells (Figure 5A). The combination of MK and RANKL also increased differentiation (Figures 5A and B). As has been reported, RANKL and M-CSF in combination also increased the number of TRAP-positive cells. The addition of MK to the combination of M-CSF and RANKL apparently increased the differentiation more than did the combination of M-CSF and RANKL, although the difference was not large enough to be significant. Since fully differentiated osteoclasts

are multinuclear, we then evaluated the effect of MK by counting TRAP-positive cells with >3 nuclei, and we reached the same conclusion (Figure 5B). The magnitude of induction by MK was greater by this assay. A photograph of TRAP staining is shown in Figure 5C.

We also investigated the osteoclast-inducing activity of MK using mouse bone marrow macrophages as the target cells (Figure 5D). In this established system, MK alone did not induce osteoclast differentiation; in the human synovial membrane, the progenitor cells might have already been at a certain stage of differentiation. As has been reported, M-CSF or RANKL alone also did not induce differentiation (36,37). MK and RANKL in combination induced differentiation, and the level of induction was similar to that by RANKL and M-CSF. The combination of MK, M-CSF, and RANKL appeared to further enhance the differentiation of osteoclasts compared with a combination of M-CSF and RANKL, but the difference was not statistically significant.

DISCUSSION

Various cytokines play different roles in the pathogenesis of RA (1). We found that MK plays roles both in the migration of inflammatory leukocytes and in the process of osteoclast differentiation. *In vitro* studies have shown that MK enhances migration of inflammatory leukocytes, both by direct action and by induction of chemokines (22–24). The activity of promoting osteoclast differentiation is important in relation to bone destruction. The activity of MK in cooperating with RANKL was as strong as that of M-CSF. This spectrum of activity is not seen in cytokines other than MK.

A virtual lack of antibody-induced arthritis in *Mdk*^{-/-} mice indicates the essential role of MK in the pathogenesis of RA. On the one hand, this resistance to the disease in an experimental model is comparable to that found in IL-1-deficient mice (7). On the other hand, TNF α -deficient mice occasionally develop experimental arthritis (7). The development of collagen-induced arthritis (5), but not that of immune complex-induced arthritis (7), is severely suppressed in IL-6-deficient mice. This is probably because IL-6 is mainly involved in lymphocyte activation. In the present study, we used the antibody-induced arthritis model, since we can exclude the effects on antibody production in this model.

The close correlation of MK with RA is also underscored by the high MK levels in the synovial fluid and sera of RA patients. More than 90% of RA patients exhibited increased serum levels of MK. Such a high

frequency of elevation associated with RA has been observed in levels of IL-6, but not in levels of IL-1 or TNF α (40). The high levels of MK expression in specimens from RA patients raised the possibility that MK levels can be used to aid screening for RA, since diagnosis of RA at an early stage requires the determination of multiple factors.

Concordant with the critical role of MK in the pathogenesis of arthritis, MK was detected in the synovial membrane both in RA patients and in mice during the process of arthritis development. We previously reported MK expression in the synovial membrane of RA patients, although cells with MK expression were not identified (24). The present study revealed MK expression both in macrophage-like cells and in fibroblast-like cells in the synovial membrane. Therefore, MK is present at the right time and place to exert the two activities related to pathogenesis of arthritis. In addition, MK secreted by activated lymphocytes may also contribute to arthritis development (41).

In addition to TNF α and IL-1, IL-6 is becoming a promising target of RA therapy (42). The findings of the present investigation suggest that MK is another candidate for therapeutic application. One important point is that MK plays critical roles in two distinct stages of RA pathogenesis. A problem in anticytokine therapy for RA is that some patients do not respond. The difference in the mechanism of action of MK compared with those of TNF α , IL-1, and IL-6 may result in a difference in the population of responding patients. Another noteworthy point is that MK is predominantly expressed during the midgestation period, and only in a restricted region in the adult (43,44). The restricted MK expression in adults may be helpful in avoiding serious side effects.

Recently, pleiotrophin, which has ~45% sequence identity with MK, has been found to be induced in the synovial membranes of RA patients (45), suggesting that pleiotrophin also contributes to the pathogenesis of RA. Although MK and pleiotrophin exhibit a similar function in many systems (14,19), the evident phenotype of *Mdk*^{-/-} in arthritis development indicates that the two factors play different roles in the process. Indeed, pleiotrophin *in vitro* acts as a growth factor for synovial cells in RA (45).

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