J. Biochem. 122, 453-458 (1997)

Midkine, a Retinoic Acid-Inducible Heparin-Binding Cytokine in Inflammatory Responses: Chemotactic Activity to Neutrophils and Association with Inflammatory Synovitis[1]

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Received for publication, April 15, 1997

Midkine (MK) is a retinoic acid-inducible heparin-binding cytokine. In the inflammatory synovitis of rheumatoid arthritis and osteoarthritis, MK was detected in synovial fluid, synoviocytes, and endothelial cells of new blood vessels. Normal synovial fluid and non-inflammatory synovial tissue did not contain detectable MK. Therefore, MK showed inflammation-associated expression in these cases. Furthermore, MK was found to promote chemotaxis of neutrophils in the range of 10 ng/ml. The mode of action of MK was found to be haptotactic; the substrate-bound form of MK was the active one. MK is also known to promote fibrinolysis. These activities of MK are in agreement with the modes of MK expression in various pathological statuses, and thus MK is proposed to be an important molecule regulating inflammatory responses.

Key words: chemotaxis, inflammation, midkine, neutrophil, synovitis.

Cytokines and growth factors are important in the control of inflammatory responses and tissue repair (*1-4*). Although the roles of certain factors in these processes have become increasingly clear, we still do not have the entire picture of the cytokine/growth factor network responsible for their control. Herein, we present evidence that midkine (MK), a cytokine with multiple activities, plays important roles in these processes.

MK is a heparin-binding protein of 13 kDa, and was found as the product of a retinoic acid-responsive gene, which becomes activated at the early differentiation stage of embryonal carcinoma cells (*5, 6*). MK is structurally unrelated to fibroblast growth factors, typical heparin-binding growth factors, and is the initial member of a new cytokine/growth factor family, which so far has only two members, MK and pleiotrophin (PTN, also called HB-GAM) (*7-11*). MK enhances the survival, differentiation and neurite outgrowth of embryonic neurons and is mitogenic to certain cell lines (*12-15*). Since its discovery, MK has been implicated in the regulation of embryogenesis, based on the finding that it is expressed in various tissues in a manner strictly controlled both spatially and temporally during embryogenesis (*16, 17*). In a healthy adult, MK is expressed only in highly restricted sites. Recently, it was found that an anti-MK antibody inhibits the differentiation of the tooth germ *in vitro* (*18*).

On the other hand, the importance of MK in tissue repair and diseases has become increasingly clear. MK expression is increased in various human carcinomas, such as stomach, colon, pancreatic, lung, breast, and hepatocellular carcinomas (*19-21*). High level expression of MK is correlated with a poor prognosis of patients with neuroblastomas (*22*). The senile plaques in Alzheimer's disease invariably accumulate MK (*23*). MK is newly expressed in the edematous region at an early stage of cerebral infarction (*24*). These findings raised the possibility that MK is involved in tissue repair and abnormalities of the latter leading to diseases. Indeed, MK prevents retinal degeneration induced by prolonged exposure to light (*25*). Recently, MK was found to enhance plasminogen activator activity in endothelial cells and to enhance fibrinolytic activity (*26*), which is important in cell migration upon inflammation, tumor invasion and angiogenesis. These findings prompted us to examine MK expression in the inflammatory states associated with rheumatoid arthritis (RA) and osteoarthritis (OA). Furthermore, we examined whether MK triggers the initial stage of inflammation, namely, the recruitment of leukocytes (*27*). Evidence obtained in this research combined with previous data enable us to propose that MK is one of the key molecules regulating the physiological and pathological processes of tissue inflammation.

MATERIALS AND METHODS

Human Synovial Fluid and Joint Biopsy Specimens—Synovial fluid was collected by aspiration from patients with inflamed synovitis accompanied by OA or RA. Synovial tissue was obtained upon total knee replacement for osteoarthritis and rheumatoid arthritis. The patients were 26-72 years of age, the average age being 52 years.

MK and Anti-MK Antibodies—Chemically synthesized

[1] This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan.
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Abbreviations: HB-GAM, heparin-binding growth-associated molecule; MK, midkine; OA, osteoarthritis; PTN, pleiotrophin; RA, rheumatoid arthritis.

human MK (*28*) was purchased from Peptide Research Institute, Mino, Osaka. Anti-human MK antibodies (*29*) and anti-mouse MK antibodies (*14*) were raised in rabbits and affinity purified as described in the respective references.

Assay for MK in Synovial Fluid—Enzyme-linked immunoassaying of MK was performed as described previously (*29*).

Western Blotting—Samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Laemmli (*30*). Proteins in the gels were transferred to nitrocellulose membranes according to the method of Towbin *et al.* (*31*). After incubation in 5% skim milk in Dulbecco's phosphate buffered saline (PBS) overnight at 4°C, the nitrocellulose sheets were incubated with diluted anti-human MK antibodies (20 μg/ml in 5% skim milk) for 2 h at room temperature. After washing with PBS containing 0.1% Tween 20, the membranes were incubated with affinity-purified anti-rabbit IgG conjugated with horseradish peroxidase (Jackson Immunoresearch Laboratories, Baltimore, USA), then stained with 4-chloro-1-naphthol.

Immunohistochemistry—Immunohistochemistry was performed as previously described (*14*). Briefly, samples were fixed in neutralized buffered formalin and subsequently embedded in paraffin. The fixed samples were sliced at 5 μm thickness. The sections were incubated overnight at 4°C with affinity-purified anti-human MK antibodies (15 μg/ml, Ref. *29*) in PBS containing 0.2% bovine serum albumin (BSA) and 2% normal goat serum. Control sections were incubated in either 2% BSA in PBS or normal rabbit serum. The sections were then incubated with biotinylated goat anti-rabbit antibodies (source dilution, 1:250 in PBS), then washed and incubated with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, USA). Peroxidase was revealed by incubation with 3-amino-9-ethylcarbazole (AEC) containing 1% H_2O_2. In control staining, the first antibody (anti-human MK antibodies) was replaced with PBS containing 0.2% BSA and 2% normal goat serum. No staining was observed in control staining.

Assay of Efficacy of MK in Promoting Migration of Human Neutrophils—Human peripheral blood from healthy donors was fractioned by Ficoll-Hypaque density gradient centrifugation to isolate neutrophils (*32*). After washing with RPMI 1640 medium, the cells were suspended at a density of 2.5×10^6 cells/ml in RPMI 1640 medium supplemented with 10% human serum from a blood group AB donor (AB serum). A typical preparation contained more than 95% viable neutrophils. The migration of neutrophils caused by MK was measured in a chemotaxicell (Kurabo, Osaka) as the upper chamber, which was identical to a Boyden chamber with an attached polycarbonate filter. As the lower chamber, a 24-well plate (3047, Falcon) was used. MK was diluted in RPMI 1640 medium containing 10% AB serum, added to the 24-well plate, and then neutrophils (5×10^5 cells) in RPMI 1640 containing 10% AB serum were added to the chemotaxicell. The chambers were placed in a humidified incubator at 37°C with 5% CO_2 for 3 h. Cells that migrated through the 5 μm filter were fixed in 100% ethanol, stained, then counted. Counts were made in ten fields (Olympus AX80 microscope, magnification ×400) for each assay, and each sample was assayed in triplicate. Data were expressed as means ± SEM. Checkerboard analysis was used to distinguish between chemotaxis (directed migration) and chemokinetics (random migration) (*33*).

The haptotaxis assay was performed to determine whether MK functions in a soluble form (chemotactic mechanism) or a substrate-bound form (haptotactic mechanism). The haptotaxis assay was conducted as described by Rot (*34*). As the first step, positive haptotactic gradients of MK were established by filling some of the bottom wells with MK (1 to 100 ng/ml) to coat the lower surface of the membrane with MK, and the corresponding top wells were filled with RPMI 1640 medium. Negative haptotactic gradients were established by filling another set of top wells with MK (1 to 100 ng per well) and the corresponding bottom wells with RPMI 1640. To examine chemotaxis, both top and bottom wells were filled with RPMI 1640 medium. After incubation at 37°C for 20 min, the chemotaxicells, which consisted of the upper chamber and the polycarbonate filter were thoroughly washed in a RPMI medium bath to remove the attractant not bound to the filter. In the second step, RPMI medium containing 10% AB serum was used to fill the bottom wells corresponding to those wells with precoated positive and negative gradients. The wells corresponding to those that did not contain attractant in the first step were used to assess chemotaxis by adding attractants (1 to 100 ng/ml) to the bottom chambers. Neutrophils (5×10^5) were placed in each top well of the second step, and their migration was measured by counting the cells that migrated across the filter during a 30 min incubation at 37°C. Counts were made in ten fields for each assay, and each sample was assayed in triplicate. All results were expressed as the number of neutrophils ± SEM.

RESULTS

MK Is Present in Synovial Fluid and Synovial Tissues

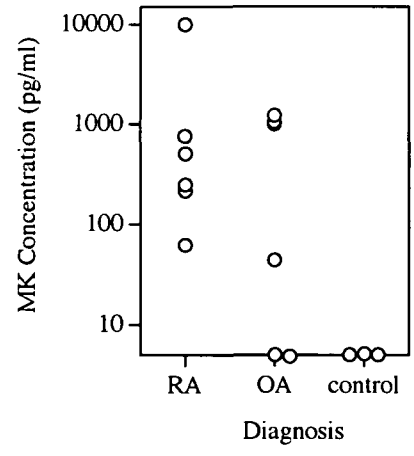

Fig. 1. **Enzyme immunoassay of MK in synovial fluid revealed detectable MK in the fluid from 10 patients with inflammatory synovitis.** In patients with rheumatoid arthritis (RA) ($n=6$), the values ranged from 62 to 10,000 pg/ml. In patients with osteoarthritis (OA) ($n=6$), values ranged from non-detectable to 1,225 pg/ml. In control synovial fluid from healthy human donors ($n=3$), MK was not detectable. The limit of detection of MK in the present assay was 9 pg/ml. For convenience, the cases with non-detectable MK are plotted at the bottom of the figure.

of Patients with Rheumatoid Arthritis and Osteoarthritis—Enzyme immunoassay detected MK in synovial fluids of patients with RA and OA. The level of MK was 44 to 10,000 pg/ml. In the synovial fluid from three normal human subjects, MK was not detected. On the other hand, in all the six patients with RA, MK was present in synovial fluid (Fig. 1). In four of six patients with OA, the synovial fluid also contained significant amounts of MK (Fig. 1). In two cases of OA, the synovial fluid was highly viscous, probably due to a high content of glycosaminoglycans, and MK was not detected before or after hyaluronidase digestion. Thus, we concluded that the presence of MK in synovial fluid is a phenomenon significantly related to the inflammatory status of synovitis.

Biopsy specimens from five patients (three cases of RA and two of OA) were analyzed histochemically for the expression of MK. All the specimens comprised hyperplastic, inflamed synovial tissue, which was characterized histologically by the proliferation of synovial lining cells, extensive infiltration of lymphocytes and macrophages, and a number of new blood vessels. In the specimens from two RA patients, the area between synovial lining cells and new blood vessels was diffusely stained by an anti-MK antibody (Fig. 2A). Interestingly, synovial lining cells (Fig. 2B) and endothelial cells of capillaries (Fig. 2C) were found to be intensely stained for MK. In one patient with RA , the staining was not as intense as in the other cases, probably because the disease was not in an active state. In two cases of an inflamed synovium with OA, the mode of immunostaining with the antibody to MK was almost the same as that in two patients with RA. Since a normal human synovium was not available for analysis, we examined biopsy specimens from a patient who had had an artificial joint replaced and who had no inflammatory synovitis; no immunostaining was detected in these specimens.

We also performed Western blot analysis of synovial tissue extracts. A high level of MK was detected in the extract from a case of active inflammatory synovitis with OA (Fig. 3, lane 1). An active inflammatory site of synovitis of RA contained a moderate level of MK (Fig. 3, lane 2), while a lower level of MK was detected in an inactive inflammatory site in the same patient (Fig. 3, lane 3). MK was not detected in the synovium from the patient with an artificial joint and no inflammatory synovitis (Fig. 3, lane 4). Therefore, the MK immunoreactive material was confirmed to be MK. Furthermore, the tendency observed on immunohistochemistry, that the intensity of MK expression was correlated with the severity of the inflammation, was also observed on Western blot analysis.

MK Is a Chemotactic Factor Acting on Neutrophils—To evaluate the role of MK in the initial stage of the inflammatory reaction, namely, the recruitment of leukocytes, we examined the chemotactic activity of MK on neutrophils. On chamber analysis, MK placed in the lower chamber was found to stimulate the migration of neutrophils placed in the upper chamber (Fig. 4). The optimum concentration of MK attracting neutrophils was 10 ng/ml ($p<0.01$) (Fig. 4).

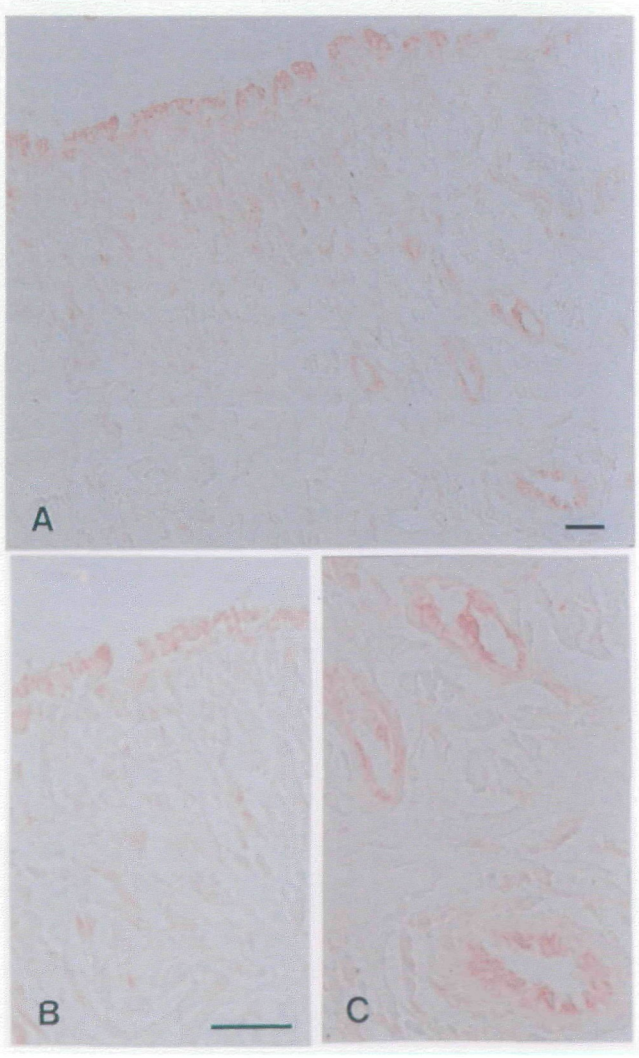

Fig. 2. **Immunohistochemical staining of inflamed synovial tissue from a patient with active inflammatory synovitis of RA.** The area between synovial lining cells and new blood vessels was stained diffusely (A). A population of cells of the synovial surface identified as synoviocytes was stained strongly for MK (B). Endothelial cells of new blood vessels were also stained with antibodies for MK (C). Bar, 50 μm.

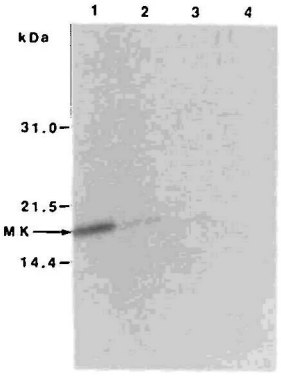

Fig. 3. **Western blot analysis of synovial tissue extracts.** Biopsy specimens from an active inflammatory synovitis case showed a high level of MK (lane 1). A moderate level of MK was expressed in an extract of an active site of inflamed synovium (lane 2), while a lower level of MK was detected at the site of an inactive inflamed synovium from the same patient (lane 3). MK was not detected in an extract of a synovium without histologically significant inflammation, which was obtained upon replacement of an artificial joint (lane 4). Note that MK migrates as an 18-kDa band, not a 13-kDa band, due to highly basic nature of the protein.

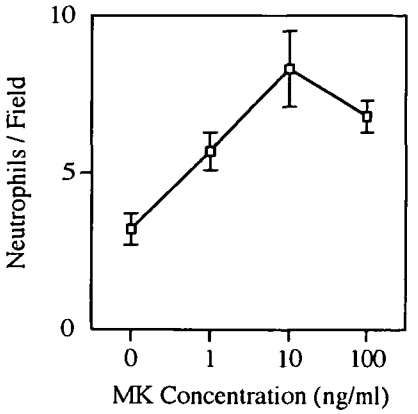

Fig. 4. **The mode of neutrophil migration in response to MK.** MK was added to the lower wells at the indicated concentrations. Points represent the number of neutrophils that had migrated to the lower surface of the filter after 3 h of incubation with MK. The results are expressed as mean migrated cells per field ($n=4$).

TABLE I. **Checkerboard analysis of neutrophil chemoattraction by MK.** Data are expressed as the number of migrated cells per field. Values represent the mean±SEM ($n=4$).

Concentration of MK in lower chamber (ng/ml)	Concentration of MK in upper chamber (ng/ml)			
	0	1	10	100
0	3.2±0.5	2.1±0.4	3.7±0.5	2.8±0.3
1	5.7±0.6	2.6±0.6	4.5±0.7	3.5±0.4
10	8.3±1.2	5.5±0.7	4.7±0.5	2.6±0.4
100	6.8±0.5	6.9±0.6	6.9±0.7	5.8±0.9

Checkerboard analysis was performed to determine whether neutrophil migration in response to MK was directed (chemotactic) or random (chemokinetic) (Table I). Migration of neutrophils across the filter occurred whenever the concentration of MK in the lower chamber was greater than that in the upper chamber, indicating that migration occurred along a concentration gradient of MK, that is, MK was chemotactic rather than chemokinetic.

Given that cell motility is an adhesion-dependent event (*27*), and MK strongly binds to syndecans (*17, 35*), a family of cell surface heparansulfate proteoglycans (*36*), we also investigated the ability of substrate-bound MK to induce neutrophil migration. To examine MK-mediated neutrophil haptotaxis, MK was precoated on polycarbonate filters, and the filters were used as chemotaxis chambers in the absence of a soluble chemoattractant. MK bound to filters even at a low concentration (1 ng/ml) ($p<0.01$) stimulated neutrophil migration after only 30 min of incubation (□, Fig. 5). In the chemotaxis assay, stimulation of neutrophil migration was not detected with various concentrations of MK after 30 min of incubation (◆, Fig. 5). Stimulation of the migration was also not observed in a negative control, in which MK was absorbed to the same side of the filter as the target cells (○, Fig. 5).

DISCUSSION

In patients with inflammatory synovitis, namely, RA and OA, MK was found to be expressed in both synoviocytes and endothelial cells of adjacent blood vessels. This inflammation-associated expression of MK was also reflected in the significant content of MK in the synovial fluid of the patients; normal synovial fluid did not contain detectable MK. This new expression of MK is likely to be involved in the etiology of synovitis, since we also found that MK is chemotactic toward neutrophils.

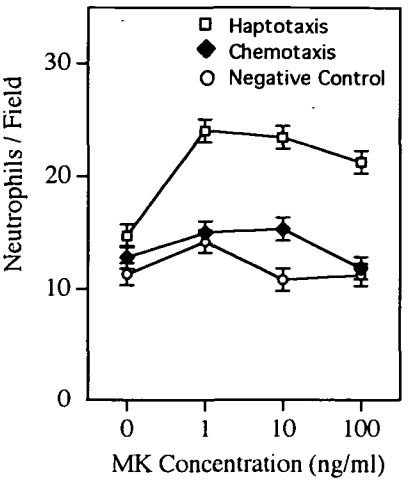

Fig. 5. **Haptotaxis as the mechanism of MK action.** Concentration-response curves for neutrophil migration to the precoated lower surface of a filter with MK for assaying of haptotaxis (□). For assaying of chemotaxis (◆), both sides of the filter were precoated, and as a negative control (○), the upper surface of the filter was precoated with MK. Points represent the numbers of neutrophils that migrated to the lower surface of the filter after 30 min incubation. The results are expressed as mean migrated cells per field ($n=4$).

The migration of neutrophils into tissues involves interaction with the vascular endothelium and subsequent migration through the underlying basement membrane, during which time the neutrophils come into close contact with extracellular matrix components and connective tissue cells (*27, 37*). The adherence of neutrophils to endothelial cells or extracellular matrices is likely to play a critical role in triggering neutrophil activation at extravascular sites of infection, inflammatory disorders, tissue damage, and remodeling.

Recently, chemoattractant factors belonging to a novel family of proinflammatory proteins were identified and classified as the chemokine family. These proteins play a key role in the orchestration of the immune response, ensuring that the correct immune effector cells are recruited and activated at the right place at the right time (*38-42*). Typical members of the chemokine family are IL-8 (an α chemokine), which is chemoattractive to neutrophils, and macrophage inflammatory protein 1β (MIP-1β; a β chemokine), which is chemoattractive to monocytes. Recent structural studies revealed that while the monomeric structures of α and β chemokines are very similar, their dimeric structures are dramatically different from each other, suggesting that this structural difference may account for the functional differences between the two families (*43*).

We could find no homology between MK and members of the chemokine family. However, both MK and chemokine family members have high contents of cysteine and basic amino acids. IL-8 is an 8-kDa protein and has three antiparallel β-sheets (*44*). MK is a 13-kDa protein and consists

of two domains (*45*), each of which has three anti-parallel β sheets (Inagaki, F., personal communication); a domain of MK exhibits overall similarity to IL-8 in the 3-D structure. In this respect, it is noteworthy that dimeric IL-8 is also active (*43*): the dimeric form of IL-8 may well have a shape similar to that of the MK monomer.

The similarity of MK and IL-8 extends to heparin-binding activity. MK strongly binds to heparin, and also to syndecans, cell-surface heparansulfate proteoglycans (*17, 35*). IL-8 also strongly binds to heparin (*46*). This heparin-binding activity is important for the haptotaxis mechanism underlying the actions of both factors, as mentioned below. For the attraction of leukocytes, chemotaxis or haptotaxis can be considered (*27*). In chemotaxis, cells move in the direction of increasing concentrations of a chemoattractant, which typically is a soluble molecule that can diffuse away from the site of its production, where its concentration is highest. An alternative mechanism to chemotaxis is haptotaxis. In haptotaxis, cells migrate to the region of highest adhesiveness. On a gradient of an adhesive ligand affixed to the surface of vascular endothelial cells or to an extracellular matrix, motile cells such as leukocytes in the peripheral bloodstream will tend to accumulate in the region of highest ligand density. While soluble cytokines would be rapidly washed away from their source by the blood flow, a more efficient system would involve the capture of secreted cytokines by receptors on endothelial cells, resulting in a high concentration of cytokines located on the endothelial cells, which would function as a chemoattractant for blood cells and would activate the migrated cells. Proteoglycans on endothelial cell surface are considered to mediate the immobilization of cytokines and growth factors, suggesting that proteoglycans play key roles in the mechanism of haptotaxis (*47, 48*).

As described above, MK resembles IL-8 in mediating chemoattraction toward neutrophils. Importantly, MK exhibits another activity related to an inflammatory response, namely, enhancement of fibrinolytic activity (*24*). To the best of our knowledge, this is the first case of a molecule exhibiting both chemotactic activity toward leukocytes and enhancement of fibrinolytic activity. For example, fibroblast growth factors enhance fibrinolytic activity (*49*) but do not exhibit chemotactic activity toward leukocytes. Chemokines such as IL-8 have chemoattractant activity toward inflammatory cells, but they have not been reported to enhance fibrinolytic activity. The concerted action of the two activities is important in the initial stage of inflammation, and will also contribute to the initiation of the pathological status. For example, MK will cause leukocyte migration in two distinct steps: one is the attraction of leukocytes through a haptotactic mechanism, and the other the infiltration of leukocytes from the bloodstream to a tissue by virtue of degradation of the basement membrane through the enhancement of fibrinolytic activity. The dual activities of MK will explain the physiological and pathological significance of the expression of MK in early stages of tissue damage and inflammation.

Finally, the chemotactic activity of MK toward neutrophils will be a key to analyze the action mechanism of MK, since the response occurs as early as in 30 min, enabling the use of various signal transduction inhibitors to dissect MK-induced cellular response.

REFERENCES

1. Damme, J.V., Proost, P., Lenaerts, J.P., and Opdenakker, G. (1992) Structural and functional identification of two human, tumor-derived monocyte chemotactic proteins (MCP-2 and MCP-3) belonging to the chemokine family. *J. Exp. Med.* **176**, 59-65
2. Fava, R.A., Oslem, N.J., Green, G.S., Yeo, K.T., Berse, B., Jackman, R.W., Senger, D.R., Dvorak, H.F., and Brown, L.F. (1994) Vascular permeability factor/endothelial growth factor (VPF/VEGF): accumulation and expression in human synovial fluids and rheumatoid synovial tissue. *J. Exp. Med.* **180**, 341-346
3. Heldin, C.H. (1992) Structural and functional studies on platelet-derived growth factor. *EMBO. J.* **11**, 4251-4259
4. Furie, M.B. and Randolph, G.J. (1995) Chemokines and tissue injury. *Am. J. Pathol.* **146**, 1287-1301
5. Kadomatsu, K., Tomomura, M., and Muramatsu, T. (1988) cDNA cloning and sequencing of a new gene intensely expressed in early differentiation stages of embryonal carcinoma cells and in mid-gestation period of mouse embryogenesis. *Biochem. Biophys. Res. Commun.* **151**, 1312-1318
6. Tomomura, M., Kadomatsu, K., Matsubara, S., and Muramatsu, T. (1990) A retinoic acid-responsive gene, MK, found in the teratocarcinoma system: heterogeneity of the transcript and the nature of the translation product. *J. Biol. Chem.* **265**, 10765-10770
7. Muramatsu, T. (1993) Midkine, the product of a retinoic acid responsive gene, and pleiotrophin constitute a new protein family regulating growth and differentiation. *Int. J. Dev. Biol.* **37**, 183-188
8. Muramatsu, T. (1994) The midkine family of growth/differentiation factors. *Dev. Growth Differ.* **36**, 1-8
9. Merenmies, J. and Rauvala, H. (1990) Molecular cloning of the 18 kDa growth associated protein of developing brain. *J. Biol. Chem.* **265**, 16721-16724
10. Li, Y.S., Milner, P.G., Chauhan, A.K., Watson, M.A., Hoffman, R.M., Kodner, C.M., Milbrandt, J., and Deuel, T.F. (1990) Cloning and expression of developmentally regulated protein that induces mitogenic and neurite outgrowth activity. *Science* **250**, 1690-1694
11. Peng, H.B., Ali, A.A., Dai, Z., Daggett, D.F., Raulo, E., and Rauvala, H. (1995) The role of heparin binding growth-associated molecule (HB-GAM) in the postsynaptic induction in cultured muscle cells. *J. Neurosci.* **15**, 3027-3038
12. Muramatsu, H. and Muramatsu, T. (1991) Purification of recombinant midkine and examination of its biological activities: functional comparison of new heparin binding factors. *Biochem. Biophys. Res. Commun.* **177**, 652-658
13. Michikawa, M., Kikuchi, S., Muramatsu, H., Muramatsu, T., and Kim, S.U. (1993) Retinoic acid responsive gene product, midkine, has neurotrophic functions for mouse spinal cord and dorsal root ganglion neurons in culture. *J. Neurosci. Res.* **35**, 530-539
14. Muramatsu, H., Shirahama, H., Yonezawa, S., Maruta, H., and Muramatsu, T. (1993) Midkine, a retinoic acid inducible growth/differentiation factor; immunochemical evidence for the function and distribution. *Dev. Biol.* **159**, 392-402
15. Nurcombe, V., Fraser, N., Herlaar, E., and Heath, J.K. (1992) MK: a pluripotential embryonic stem-cell-derived neuroregulatory factor. *Development* **116**, 1175-1183
16. Kadomatsu, K., Huang, R.P., Suganuma, T., Murata, F., and Muramatsu, T. (1990) A retinoic acid responsive gene MK found in the teratocarcinoma system is expressed in spatially and temporally controlled manner during mouse embryogenesis. *J. Cell Biol.* **110**, 607-616
17. Mitsiadis, T.A., Salmivirta, M., Muramatsu, T., Muramatsu, H., Rauvala, H., Lehtonen, E., Jalkanen, M., and Thesleff, I. (1995) Expression of the heparin-binding cytokines, midkine (MK) and HB-GAM (pleiotrophin) is associated with epithelial-mesenchymal interactions during fetal development and organogenesis. *Development* **121**, 37-51
18. Mitsiadis, T.A., Muramatsu, T., Muramatsu, H., and Thesleff, I.

(1995) Midkine (MK), a heparin-binding growth/differentiation factor, is regulated by retinoic acid and epithelial-mesenchymal interactions in the developing mouse tooth, and affects cell proliferation and morphogenesis. *J. Cell Biol.* **129**, 267-281
19. Tsutsui, J., Kadomatsu, K., Matsubara, S., Nakagawara, A., Hamanoue, M., Takao, S., Shimazu, H., Ohi, Y., and Muramatsu, T. (1993) A new family of heparin-binding growth/differentiation factor: increased midkine expression in Wilm's tumor and other human carcinomas. *Cancer Res.* **53**, 1281-1285
20. Aridome, K., Tsutsui, J., Takao, S., Kadomatsu, K., Ozawa, M., Aikou, T., and Muramatsu, T. (1995) Increased midkine gene expression in human gastrointestinal cancers. *Jpn. J. Cancer Res.* **86**, 655-661
21. Garver, R.I., Radford, D.M., Donis-Keller, H., Wick, M.R., and Milner, P. (1994) Midkine and pleiotrophin expression in normal and malignant breast tissue. *Cancer* **74**, 1584-1590
22. Nakagawara, A., Milbrabdt, J., Muramatsu, T., Deuel, T.F., Zhao, H., Cnaan, A., and Brodeur, G.M. (1995) Differential expression of pleiotrophin and midkine in advanced neuroblastomas. *Cancer Res.* **55**, 1792-1797
23. Yasuhara, O., Muramatsu, H., Kim, S.U., Muramatsu, T., Murata, H., and McGeer, P.L. (1993) Midkine, a novel neurotrophic factor, is present in senile plaques of Alzheimer disease. *Biochem. Biophys. Res. Commun.* **192**, 246-251
24. Yoshida, Y., Goto, M., Tsutsui, J., Ozawa, M., Sato, E., Osame, M., and Muramatsu, T. (1995) Midkine is present in the early stage of cerebral infarct. *Dev. Brain Res.* **85**, 25-30
25. Unoki, K., Ohba, N., Arimura, H., Muramatsu, H., and Muramatsu, T. (1994) Rescue of photoreceptors from the damaging effects of constant light by midkine, a retinoic acid responsive gene product. *Invest. Ophthalmol. Vis. Sci.* **35**, 4063-4068
26. Kojima, S., Muramatsu, H., Amanuma, H., and Muramatsu, T. (1995) Midkine enhances fibrinolytic activity of bovine endothelial cells. *J. Biol. Chem.* **270**, 9590-9596
27. Springer, T.A. (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm. *Cell* **76**, 301-314
28. Inui, T., Bodi, J., Kubo, S., Nishio, H., Kimura, T., Kojima, S., Maruta, H., Muramatsu, T., and Sakakibara, S. (1996) Solution synthesis of human midkine, a novel heparin binding neurotrophic factor consisting of 121 amino acid residues with five disulphide bonds. *J. Pept. Sci.* **2**, 28-39
29. Muramatsu, H., Song, X., Koide, N., Hada, H., Tsuji, T., Kadomatsu, K., Inui, T., Kimura, T., Sakakibara, S., and Muramatsu, T. (1996) Enzyme-linked immunoassay of midkine and its application in elevating midkine levels in the developing mouse brain and in sera from patients with hepatocellular carcinoma. *J. Biochem.* **119**, 1171-1175
30. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
31. Towbin, H., Staehelin, T., and Gorden, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354
32. Venaille, T.J., Misso, N.L.A., Phillips, M.J., Robinson, B.W.S., and Thompson, P.J. (1994) Effects of different density gradient separation techniques on neutrophil function. *Scand. J. Clin. Lab. Invest.* **54**, 385-391
33. Zigmond, S.H. and Hirsch, J.G. (1973) Leukocyte locomotion and chemotaxis. *J. Exp. Med.* **137**, 387-410
34. Rot, A. (1993) Neutrophil attractant/activation protein-1(interleukin-8) induces in vitro neutrophil migration by haptotactic mechanism. *Eur. J. Immunol.* **23**, 303-306
35. Nakanishi, T., Kadomatsu, K., Okamoto, T., Ichihara, K., Kojima, T., Saito, H., Tomoda, Y., and Muramatsu, T. (1997) Expression of syndecan-1 and -3 during embryogenesis of the central nervous system in relation to binding with midkine. *J. Biochem.* **121**, 197-205
36. Elenius, K., Vainio, S., Laato, M., Salmivirta, M., Thesleff, I., and Jalkanen, M. (1991) Induced expression of syndecan in healing wounds. *J. Cell Biol.* **114**, 585-595
37. Folkman, J. and Shing, Y. (1992) Angiogenesis. *J. Biol. Chem.* **267**, 10931-10934
38. Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996) Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* **382**, 635-638
39. Tashiro, K., Tada, H., Heilker, R., Shirozu, M., Nakano, T., and Honjo, T. (1993) Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. *Science* **261**, 600-603
40. Schall, T.J., Bacon, K., Camp, R.D.R., Kaspari, J.W., and Goeddel, D.V. (1993) Human macrophage inflammatory protein α (MIP-1α) and MIP-1β chemokines attract distinct population of lymphocytes. *J. Exp. Med.* **177**, 1821-1825
41. Hosaka, S., Akahoshi, T., Wada, C., and Kondo, H. (1994) Expression of the chemokine superfamily in rheumatoid arthritis. *Clin. Exp. Immunol.* **97**, 451-457
42. Baggiolini, M. and Dahinden, C.A. (1994) CC chemokines in allergic inflammation. *Immunol. Today* **15**, 127-133
43. Clore, G.M. and Gronenborn, A.M. (1995) Three-dimensional structures of α and β chemokines. *FASEB J.* **9**, 57-62
44. Clark-Lewis, I., Kim, K.S., Rajarathnam, K., Gong, J.H., Dewald, B., Moser, B., Baggiolini, M., and Sykes, B.D. (1995) Structure-activity relationships of chemokines. *J. Leukoc. Biol.* **57**, 703-711
45. Fabri, L., Maruta, H., Muramatsu, H., Muramatsu, T., Simpson, R.J., Burgess, A.W., and Nice, E.C. (1993) Structural characterization of native and recombinant forms of the neurotrophic cytokine MK. *J. Chromatogr.* **646**, 213-225
46. Webb, L.M., Ehrengruber, M.U., Lewis, I.C., Baggiolini, M., and Rot, A. (1993) Binding to heparan sulfate or heparin enhances neutrophil responses to interleukin 8. *Proc. Natl. Acad. Sci. USA* **90**, 7158-7162
47. Tanaka, Y., Adams, D.H., and Shaw, S. (1993) Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. *Immunol. Today* **14**, 111-115
48. Schlessinger, J., Lax, I., and Lemmon, M. (1995) Regulation of growth factor activation by proteoglycans: what is the role of the low affinity receptors? *Cell* **83**, 357-360
49. Presta, M., Moscatelli, D., Joseph-Silverstein, J., and Rifkin, D.B. (1986) Purification from a human hepatoma cell line of a basic fibroblast growth factor-like molecule that stimulates capillary endothelial cell plasminogen activator production, DNA synthesis, and migration. *Mol. Cell. Biol.* **6**, 4060-4066