J. Biochem. 119, 1150-1156 (1996)

Midkine, a Heparin-Binding Growth/Differentiation Factor, Exhibits Nerve Cell Adhesion and Guidance Activity for Neurite Outgrowth *In Vitro*[1]

Norio Kaneda,*[,2] Amjad H. Talukder,* Hiroko Nishiyama,† Shinichi Koizumi,† and Takashi Muramatsu*

**Department of Biochemistry, Nagoya University School of Medicine, Showa-ku, Nagoya 466; and †Bio-Organics Research Department, International Research Laboratories, Ciba-Geigy Japan Ltd., Takarazuka 665*

Received for publication, January 26, 1996

By means of a baculovirus expression system, a large amount of mouse midkine (MK) was produced. The protein was purified to homogeneity by heparin-Sepharose column chromatography. The purified protein was of a mature type; the signal peptide was cleaved at the expected site. To examine the neurite-guiding activity of MK, rat embryonic brain cells (embryonic days 17-18) were cultured on plates coated with purified MK in a grid pattern. The cells attached to and extended their neurites along the substrate pattern. This interaction was strongly inhibited by heparin, but not by other glycosaminoglycans. Treatment of the cells with heparitinase was effective for inhibiting their adhesion to the substrate. These data suggest that the heparin-like domain on cell surface heparan sulfate proteoglycan is the primary site for MK binding upon interaction with nerve cells.

Key words: baculovirus, guidance molecule, heparin-binding protein, midkine, neurite outgrowth.

During development of the central and peripheral nervous systems, neuronal precursor cells undergo several steps including proliferation, differentiation, and migration, followed by neurite outgrowth and synaptic connection. Many factors, *i.e.* neurotrophins, neurite outgrowth/inhibitory factors, and cell adhesion molecules, may play pivotal roles in these steps. Although the mechanisms underlying neurite outgrowth and axonal guidance in developing neurons are not known in detail, at least two mechanisms are thought to be involved; one comprises a gradient of chemoattractant or chemorepellent molecules, and the other the expression of specific cell adhesion molecules (*1, 2*). Various cell adhesion molecules found on the cell surface membrane and in the extracellular matrix are known to possess neurite outgrowth promoting activity (*3-7*).

Midkine (MK), which was originally identified as a product of a retinoic acid inducible gene in a teratocarcinoma cell line, is a heparin-binding basic protein of M_r 13,000 (*8, 9*). MK is rich in basic amino acids and cysteine residues, all of which are crosslinked through intramolecular disulfide bridges (*10*). On comparison of the primary structures of MK and subsequently found pleiotrophin (PTN, also known as heparin-binding growth-associated molecule) (*11-13*), it was found that 45% of their amino acid residues are homologous, including the 10 cysteine residues, and that they constitute a new family of heparin-binding growth/differentiation factors (*14, 15*). The expression of MK mRNA during mouse development is strictly regulated. Its expression is highest in the midgestational period and thereafter restricted predominantly to the kidney in adulthood (*8, 16*). In accordance with their developmentally regulated expression, MK and PTN were verified to be important molecules involved in organogenesis (*17*). In the developing brain, an immunohistochemical study revealed that MK and PTN were expressed in radial glial processes as well as the ventricular zone of the cerebral cortex (*18*).

Regarding biological activity toward neurons, it was shown that MK acts as both a diffusible factor and a non-diffusible substrate molecule. As a soluble factor, a low concentration of MK promoted the survival of mouse embryonic spinal cord, dorsal root ganglion, and mesencephalic neurons in culture (*19, 20*). On the other hand, when the culture dish was coated with MK, it was demonstrated that MK promoted the neurite outgrowth and survival of rat embryonic brain cells (*21, 22*). In these studies, we used MK produced by L cells which had been transformed stably with an expression vector. To investigate the interaction between nerve cells and MK as a substrate in more detail, we needed a relatively large amount of MK. Therefore, we established a recombinant baculovirus clone which expresses a larger amount of MK in the culture medium than L cells. The MK protein was purified to homogeneity by heparin-Sepharose column chromatography. Using the purified MK, we demonstrate here that embryonic neurons

[1] This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed to the present address at Department of Biological Function and Analysis, Faculty of Pharmacy, Meijo University, Nagoya 468. Phone: +81-52-832-1781 (Ext. 330), Fax: +81-52-834-8780, E-mail: nkaneda@meijo-u.ac.jp

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MALDI/TOF, matrix-assisted laser desorption ionization/time-of-flight; MK, midkine; PBS, phosphate-buffered saline; pCMBS, *p*-chloromercuribenzene sulfonate; PTN, pleiotrophin.

preferentially attach to MK and extend their neurites along tracks of MK, suggesting the possible contribution of MK in *in vivo* axonal guidance through regulated expression of MK during development.

MATERIALS AND METHODS

Isolation of a Recombinant Baculovirus Clone Carrying Mouse MK cDNA—The full-length MK cDNA with *Bam*HI and *Pst*I sites at its 5′ and 3′ ends, respectively, was produced by PCR using mouse MK-2 cDNA (*8, 9*) subcloned into pUC18 as a template. The sense primer was designed so that 5′ untranslated region upstream from the initial methionine was removed. The antisense primer was located at 14 nucleotides downstream from the polyA$^+$ signal. The sequences of the primers were as follows, 5′ATGGATCCATGCAGCACCGAGGCTTCTTC3′ and 5′-CGCTGCAGGGAAAAAAGTGAGTTTATTTT3′, respectively. After digestion of the PCR product with *Bam*HI and *Pst*I, and purification by agarose gel electrophoresis, the resulting DNA fragment was subcloned into the *Bam*HI and *Pst*I sites of the transfer vector, pVL1393, under the control of the *Autographa californica* nuclear polyhedrin promoter (Pharmingen, CA). For the generation of a recombinant virus, *Spodoptera frugiperda* Sf-21 cells were cotransfected with 20 ng of a BaculoGold linearized virus DNA with a lethal deletion (Pharmingen, CA) and 1 μg of the recombinant transfer vector by the lipofectin (Life Technologies, MD) method in ExCell 400 serum-free medium (JRH Biosciences, KS). After incubation for 3 days at 27°C, recombinant viruses released into the medium were purified by the plaque method (*23*). The resulting clones were amplified 3 times to obtain high titer viral stocks in Grace's medium supplemented with lactalbumin hydrolysate (3.33 g/liter) and yeastolate (3.33 g/liter) (Life Technologies, MD) containing 10% fetal bovine serum (FBS).

Expression and Purification of Mouse MK—For the production of the MK protein, *Trichoplusia ni* High Five cells (Invitrogen, CA) were used. The cells were infected with the recombinant virus and then cultured in ExCell 400 for 3 days at 27°C. Then, a protease inhibitor, 0.5 mM *p*-chloromercuribenzene sulfonate (pCMBS), was added, and the culture was continued for one more day. The addition of pCMBS was effective in protecting the product from proteolytic degradation, as described previously (*24*).

The culture medium was centrifuged at 7,000 rpm for 10 min, followed by ultracentrifugation at 35,000 rpm (100,000 × *g*) for 30 min to remove virus particles. The supernatant (500 ml) obtained was directly applied on a Hi-Trap Heparin column (column size, 1 ml; Pharmacia Biotech, Uppsala), which had been equilibrated with 50 mM sodium phosphate buffer, pH 6.8, containing 0.2 M NaCl, at the flow rate of 0.5 ml/min using an FPLC system (Pharmacia Biotech, Uppsala). After washing with the same buffer, proteins were eluted stepwisely with 50 mM sodium phosphate buffer, pH 6.8, containing 0.5, 0.7, 1, and 2 M NaCl at the flow rate of 0.5 ml/min. The MK protein was eluted at the 1 M NaCl step as a single peak.

Assay for Neurite Outgrowth on MK Tracks—Rat embryonic cerebral cortex (embryonic days 17-18) was isolated. After removal of the meninges, the tissue was minced and digested with 0.25% trypsin (Life Technologies, MD) and 0.01% DNase I (Sigma Chemicals, MO) for 30 min at 37°C in phosphate-buffered saline (PBS). At the end of the incubation, the medium was changed to a culture medium comprising Dulbecco's modified Eagle's medium (DMEM) containing 0.1% FBS, an insulin/transferrin/sodium selenite mixture (Becton Dickinson, NJ), and penicillin (100 U/ml)/streptomycin (100 μg/ml) (Life Technologies, MD), followed by gentle trituration with a pasteur pipette. Remaining cell clumps and debris were removed. Single cells were collected by centrifugation at 800 rpm for 5 min and suspended in the medium described above.

The formation of a pattern of MK on a plastic culture plate was performed according to the method of Rauvala *et al.* (*25*). A culture plate (24 wells, Falcon 3057; Becton Dickinson, NJ) was coated with purified MK or poly L-lysine (Sigma Chemicals, MO) at a concentration of 10 μg/ml for 2 h at room temperature. After washing twice with H_2O, the plate was dried, and metal electron microscopy grids (HDL 200; Veco, Amsterdam) were placed in the wells, followed by irradiation with UV light at 315 nm for 30 min in a UV chamber (GS Gene Linker; Bio-Rad, CA) to produce a grid pattern of the substrate. After removing the grids, the wells were incubated with DMEM containing 10 mg/ml of BSA for 30 min at 37°C. After washing twice with H_2O, the rat brain nerve cell suspension described above was seeded (0.8×10^6 cells/well), followed by culturing for 24-48 h at 37°C under an atmosphere of 5% CO_2 and 95% air.

In experiments to analyze the effects of glycosaminoglycans, brain cells were cultured in the presence of various concentrations of glycosaminoglycans. They included heparin purified from porcine intestine (Wako Chemicals, Tokyo), heparan sulfate from bovine kidney, chondroitin sulfate A from whale cartilage, chondroitin sulfate C from shark cartilage, dermatan sulfate from porcine skin, keratan polysulfate from shark cartilage, and hyaluronic acid from porcine skin (Seikagaku Corporation, Tokyo).

In some experiments, heparitinase or heparinase, both from *Flavobacterium heparinum* (Seikagaku, Tokyo), was added to the cell suspension, followed by culturing as described above.

Analytical Procedures—SDS-PAGE at 12.5% gel concentration was carried out by the method of Laemmli (*26*), and the gel was stained with silver reagent, or subjected to Western blotting (*27*) using a specific anti-MK antiserum previously described (*22*). The protein concentration was measured by means of the micro BCA assay (Pierce, IL) using BSA as a standard. For determination of the amino acid sequence, the purified MK was applied on a reverse phase HPLC column (Nucleosil 7C18, 4.6 × 250 mm; Chemco, Osaka), and eluted with a linear gradient of acetonitrile (10-40%) in 0.1% trifluoroacetic acid to remove salts. An aliquot (1.2 nmol) of the protein was applied to a gas-phase protein sequencer (373A; Perkin Elmer Applied Biosystems, CA). For determination of the total mass of MK, the purified protein was subjected to matrix-assisted laser desorption ionization/time-of-flight (MALDI/TOF) mass spectrometry (Voyager RP; PerSeptive Biosystems, MA).

RESULTS

Production and Characterization of Recombinant Mouse MK—A full-length mouse MK cDNA including signal peptide was subcloned into a transfer vector, pVL1393, and then cotransfected into Sf-21 cells with a BaculoGold genomic DNA. From the culture medium of cotransfected cells, several recombinant viruses were isolated. The expression efficiencies of these clones were comparable, and one of the clones (M-7) was subjected to amplification on a large scale. Comparison of the expression levels in different insect cell lines, Sf-21 and High Five cells, revealed that High Five cells expressed the MK protein much more (about 5-fold) efficiently than Sf-21 cells. In addition, proteolytic degradation of MK was almost negligible in High Five cells, whereas in Sf-21 cells degradation was obvious. Furthermore, the small amount of proteolysis observed in High Five cells was completely inhibited by the addition of 0.5 mM pCMBS. Figure 1 shows the elution profile of MK from a heparin-Sepharose column. MK was eluted at the concentration of 1 M NaCl as a single peak. The purity of this fraction was examined by SDS-PAGE and silver staining. As shown in Fig. 2, MK was found to be purified to homogeneity by a single column chromatography step from the culture medium. The purified MK gave a single band corresponding to an apparent M_r of about 17,000 on both silver staining and immunoblotting. To determine whether or not the signal peptide was removed properly from the precursor protein, the N-terminal sequence of the purified preparation was determined. As shown in Fig. 3, the signal peptide was cleaved at the expected site, which coincides with the empirical rule derived from many known signal sequences (*28*). To explain the higher M_r than the value (about 13,000) calculated from the nucleotide sequence, the total mass of the purified MK was determined by MALDI/TOF mass spectrometry (Fig. 4). The m/z value of the parent peak was 13,073.3, which was in good agreement with the theoretical value of 13,070.4, indicating that the purified MK had an intact structure without any modifications. Thus, the apparent higher M_r on SDS-PAGE may be due to the high positive charge content of the sequence. Using this expression system, at least 2 mg of purified protein was obtained from 1 liter of culture medium.

Neurite Outgrowth on MK Tracks—To study the biological activity of the purified MK toward nerve cells, a neurite outgrowth assay on a substrate pattern was performed. The grid pattern of MK on culture plates was made using electron microscopy grids and the UV-inactivation technique. When rat embryonic brain cells were cultured on the MK pattern, the cells preferentially attached to the area which had been protected from UV-inactivation (Fig. 5A). When seeded at a lower cell density, clear neurite outgrowth was observed along the tracks of MK, but not in the inactivated areas (Fig. 5B). The effect of poly L-lysine seemed to be weak compared to that of MK, the cells aggregating with each other (Fig. 5C). These data show that

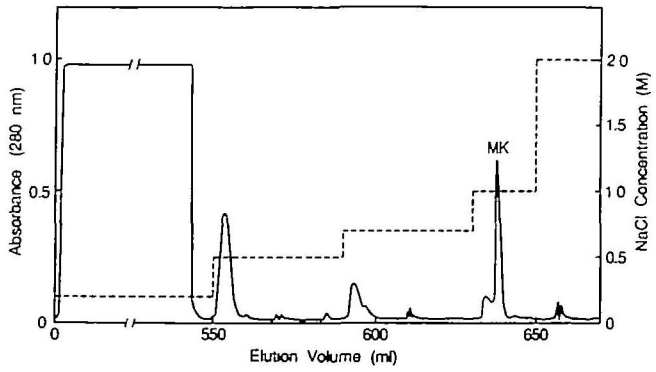

Fig. 1 **Purification of MK by heparin-Sepharose chromatography.** Culture medium from High Five insect cells (*ca* 500 ml) was ultracentrifuged at 35,000 rpm (100,000 × *g*) for 30 min, and then applied to a column of Hi-Trap Heparin (column size, 1 ml), which had been equilibrated with 50 mM sodium phosphate buffer, pH 6.8, containing 0.2 M NaCl Elution was carried out stepwise with the same buffer containing 0.2, 0.5, 0.7, 1 0, and 2.0 M NaCl at the flow rate of 0.5 ml/min.

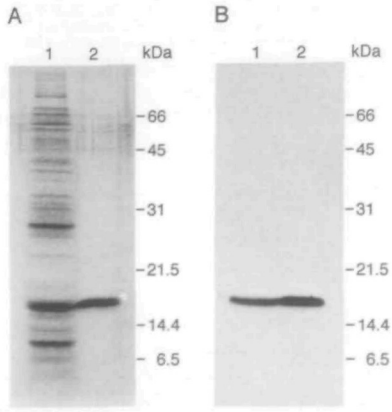

Fig. 2 **SDS-PAGE and Western blot analyses of culture medium and purified MK.** Culture medium (6 μl) or the purified MK fraction (0 4 μl) eluted from a heparin-Sepharose column shown in Fig. 1 was subjected to 12 5% SDS-PAGE, followed by silver staining (A) or Western blot analysis using anti-MK antiserum (B) Lane 1, culture medium, lane 2, purified MK The faint bands between 45 and 66 kDa in panel A are non-specific ones often observed during silver staining. The molecular masses of the standard markers are shown on the right in kDa

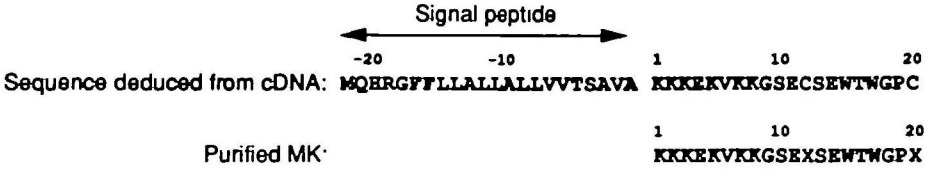

Fig. 3 **N-Terminal amino acid sequence of the purified MK.** The purified MK (1.2 nmol) was applied to a gas-phase protein sequencer (373A, Perkin Elmer Applied Biosystems). The observed sequence is aligned with the amino acid sequence deduced from the nucleotide sequence. Amino acids are shown in a one letter code, in which X represents an unidentified residue.

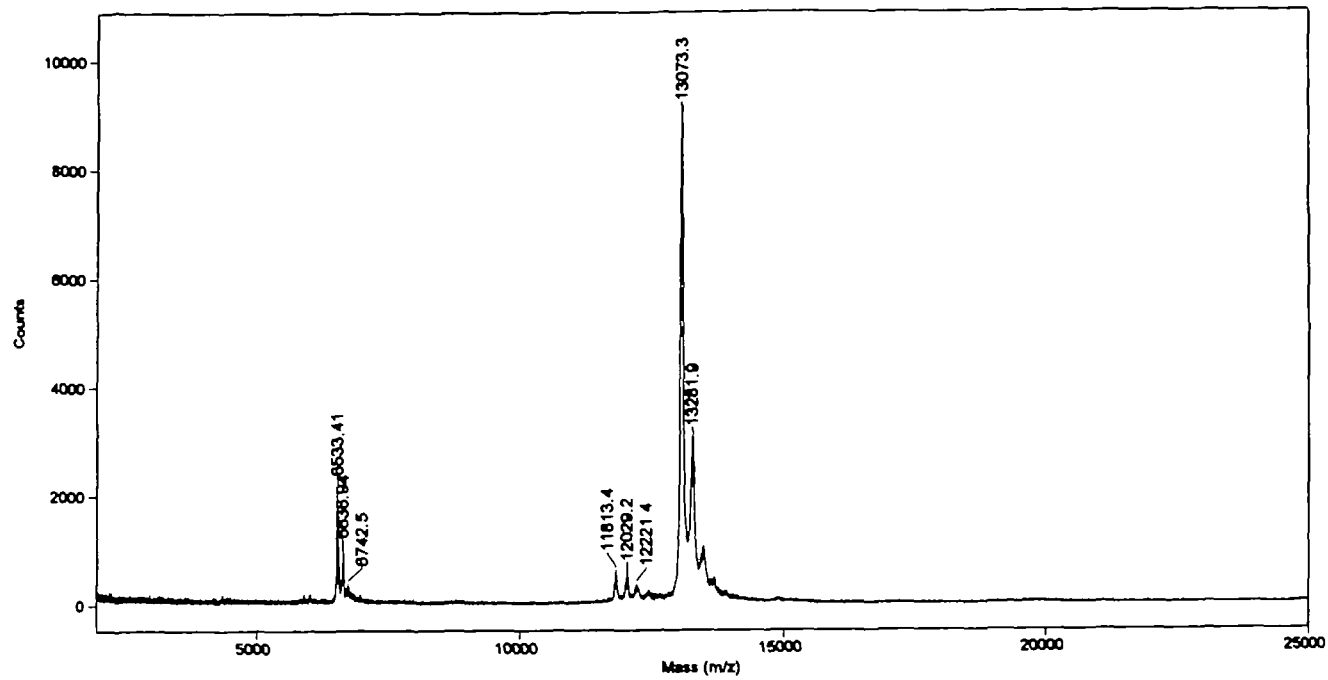

Fig. 4. **Determination of the total mass of the mouse MK by mass spectrometry.** The purified MK (7.5 pmol/0.5 μl) was subjected to MALDI/TOF mass spectrometry (Voyager RP; PerSeptive Biosystems), using 0.3% sinapinic acid dissolved in 9% acetonitrile and 0.03% trifluoroacetic acid as a matrix solution. The system was calibrated using parathyroid hormone (m/z=9,425.75) and myoglobin (m/z= 16,951.75) as external standards.

TABLE I. **Effects of various glycosaminoglycans on nerve cell adhesion.** Rat embryonic nerve cells were cultured on plates coated with a pattern of MK (10 μg/ml). Cell adhesion was evaluated after 24 h. ++, cells detached from the plate and aggregated; +, cells attached to the plate, but no grid pattern was observed; −, no inhibition.

Glycosaminoglycans	Concentration (μg/ml)					
	0.16	0.6	2.5	10	50	200
Heparin	−	+	++	++	++	++
Heparan sulfate	−	−	−	−	−	−
Chondroitin sulfate A	−	−	−	−	−	−
Chondroitin sulfate C	−	−	−	−	−	−
Dermatan sulfate	−	−	−	−	−	−
Keratan polysulfate	−	−	−	−	−	−
Hyaluronic acid	−	−	−	−	−	−

MK is one of the most effective substrates for nerve cells, and exhibits guiding activity, as shown by their preferential adhesion and increased outgrowth of neurites.

Effects of Heparin and Other Glycosaminoglycans on Nerve Cell Adhesion—MK is a heparin-binding protein, and is thought to be associated *in vivo* with the carbohydrate moiety of heparan sulfate proteoglycans on the cell surface or in the extracellular matrix. To characterize the interaction of MK with nerve cells, the effect of heparin was examined. As shown in Fig. 6, A-C, and Table I, heparin showed inhibition of nerve cell adhesion at the concentration of 0.6 μg/ml. At higher concentrations than 2.5 μg/ml, nerve cells become completely detached from the plate and aggregated with each other, and consequently no neurite outgrowth was observed. The effects of other glycosaminoglycans in this system were examined and the results are summarized in Table I. Heparan sulfate purified from bovine kidney showed no inhibitory effect, even at the highest concentration examined (Fig. 6D). Chondroitin sulfates A and C, dermatan sulfate, keratan polysulfate, and hyaluronic acid also had no effect. Thus, the effect of heparin was highly specific among the glycosaminoglycans examined, suggesting that cell surface heparin-like polysaccharide structure may be involved in the interaction of nerve cells with coated MK.

Effects of Heparitinase and Heparinase on Nerve Cell Adhesion—To determine whether or not cell surface heparan sulfate proteoglycans are directly involved in the nerve cell attachment to the coated MK, heparitinase, or heparinase was added to the culture. As shown in Fig. 6, E and F, heparitinase treatment effectively inhibited the nerve cell adhesion at the concentration of 20 mU/ml, while heparinase had almost no effect when added at the same concentration.

DISCUSSION

The present paper described the production of MK with a baculovirus system and its biological activity toward cultured embryonic neurons. The intactness of the purified protein was confirmed by N-terminal amino acid sequencing and mass spectrometry. A yield of at least 2 mg of purified MK/liter of culture medium was obtained, which was much larger than that obtained with an L cell system (about 200 μg/liter) (*21*). The large amount of MK enabled us to analyze its nerve cell adhesion activity leading to axonal guidance, especially in terms of glycosaminoglycans involved in the interaction.

When embryonic neurons were cultured on a plate with a pattern of MK, nerve cells preferentially attached to the

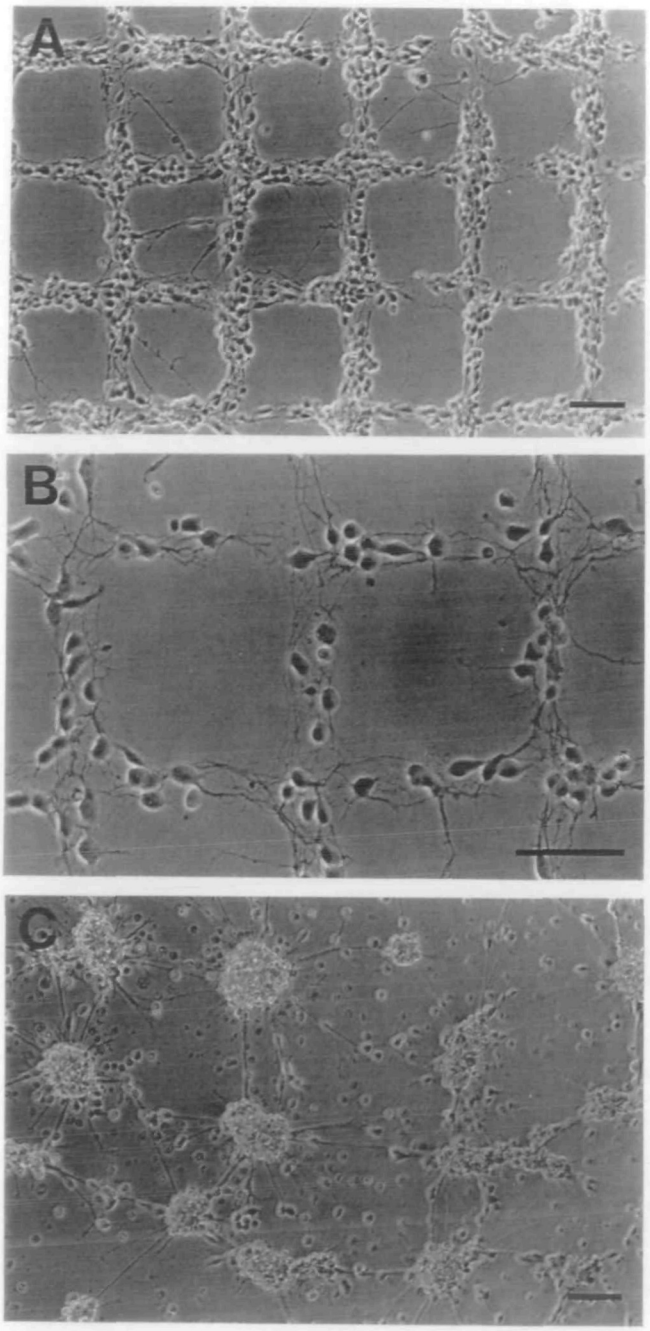

Fig. 5 **Phase-contrast photomicrographs of rat embryonic neurons cultured on a pattern of MK (A, B) or poly L-lysine (C).** Plastic culture plates (24-wells) were coated with MK or poly L-lysine (10 μg/ml) for 2 h at room temperature. A grid pattern was then made by the UV-inactivation technique using electron microscopy grids with a pitch of 125 μm (*35*). After blocking with 10 mg/ml of BSA, brain cells prepared from rat embryo cerebral cortex (embryonic days 17-18) were cultured at 37°C in a medium comprising DMEM, 0.1% FBS, insulin/transferrin/sodium selenite, and penicillin/streptomycin, under an atmosphere of 5% CO_2 and 95% air. Photographs were taken after 20 h. For details, see "MATERIALS AND METHODS." A and C: 0.8×10^6 cells/well, B: 0.2×10^6 cells/well. Bar, 50 μm

area coated with MK, and neurite outgrowth was observed exclusively along the tracks. It is believed that the growth cone of neurites selects the direction in which it should extend by detecting a more preferential one at the boundary of a substrate (*29*). The present study showed that MK is one of the most preferential substrates for neurons and that their neurites are guided according to the pattern of MK. So far, the relevance of several cell adhesion molecules to neuronal guidance activity has been elucidated (*2, 6*). For example, neural cell adhesion molecule or *N*-cadherin, typical cell adhesion molecules, may play an important role in neuronal outgrowth guidance. However, the specificities of these molecules in the nervous system cannot be defined since they are widely expressed in many tissues during development (*1*). We previously demonstrated the restricted expression of MK and PTN in the developing brain using immunohistochemistry (*18*). Both MK and PTN are significantly expressed in the radial glial processes and ventricular zone of the developing cerebral cortex. In this context, PTN has also been reported to exhibit neurite outgrowth activity *in vitro* and suggested to be involved in axonal guidance *in vivo* (*25*). Since the expression of PTN in the brain increases in the perinatal period when that of MK is declining, MK and PTN might have partly the same functions, such as in cell migration or axonal guidance, but could have their own roles as to specific neurons in the embryonic brain.

It was shown that nerve cell adhesion on a pattern of MK was strongly inhibited by low concentrations of heparin, but not by other glycosaminoglycans, and that heparitinase treatment of nerve cells significantly reduced the cell adhesion. Although heparin is known to interact with MK, these data indicate the biological significance of the interaction and strongly suggest that the highly sulfated heparin-like domain on a heparan sulfate chain is involved in the interaction of MK with nerve cells. In fact, several other heparin-binding growth factors, such as acidic and basic fibroblast growth factors, and hepatocyte growth factor, have been proved to be associated with a specific site on a heparan sulfate carbohydrate chain (*30-34*). Especially in the case of PTN, syndecan-3, a cell surface proteoglycan, has been proposed to be a receptor site for the ligand in PTN-dependent neurite outgrowth (*35*). In the present study, however, heparan sulfate purified from bovine kidney did not exhibit any inhibitory effect, even at the highest concentration of 200 μg/ml (Table I). This may imply that the heparan sulfate from bovine kidney is structurally distinct from that present in embryonic neurons; probably the latter is much more highly sulfated and/or has structural features quite unique to the embryonic brain.

The results of heparitinase and heparinase treatment seem to be rather contradictory. Heparitinase exhibits specificity toward a non-sulfated or less sulfated heparan sulfate backbone, and heparinase toward a highly sulfated (2-*O*-sulfated iduronic acid and 6-*O*- and *N*-sulfated glucosamine residues) disaccharide unit (*36, 37*). The fact that heparitinase treatment had an inhibitory effect, while heparinase had almost no effect, gave rise to the possibility that the heparan sulfate on the nerve cell surface has rather unique structural features compared to a typical haparan sulfate, such as that found in bovine kidney. If we consider the structural microheterogeneity found in heparan sulfate and heparin (*38, 39*), the above result may be attributed to the specificity of the enzyme, including the size of an aglycon or to steric hindrance. A study on structural

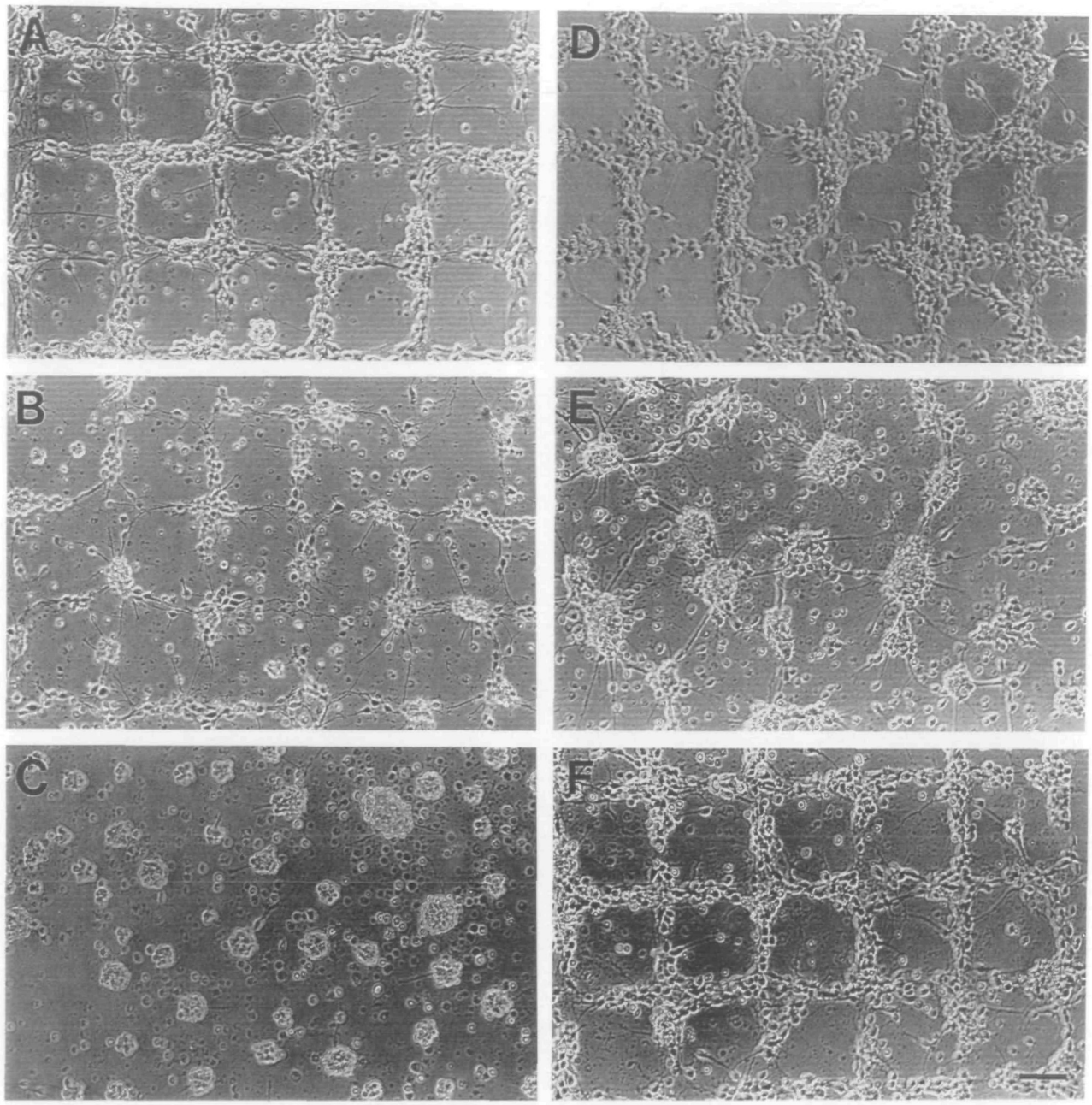

Fig. 6 **Effects of heparin, heparan sulfate, heparitinase, and heparinase on nerve cell adhesion.** The preparations of rat embryonic neurons and the pattern of MK on culture plates were the same as those described in the legend to Fig 5, and under "MATERIALS AND METHODS." The effect of heparin was examined at the concentrations of 0 16 μg/ml (A), 0 6 μg/ml (B), and 2 5 μg/ml (C) Heparan sulfate purified from bovine kidney was added at 200 μg/ml (D). Heparitinase (E) or heparinase (F) was added at the concentration of 20 mU/ml, respectively Phase-contrast photomicrographs were taken after culturing for 20 h. Bar, 50 μm.

characterization of the heparin-like domain of heparan sulfate involved in the interaction is now in progress. Further study on the isolation and structural determination of a specific oligosaccharide with inhibitory activity may facilitate understanding of the interaction between MK and nerve cells. The production of a large amount of recombinant MK reported here will be extremely useful for such studies.

We are grateful to PerSeptive Biosystems, Tokyo Branch, for the MALDI/TOF mass spectrometric measurements, and to Ms K. Yoshioka and Ms. K. Saito for their secretarial assistance.

REFERENCES

1. Dodd, J. and Jessell, T.M. (1988) Axon guidance and the patterning of neuronal projections in vertebrates. *Science* **242**, 692-699
2. Doherty, P. and Walsh, S.F (1989) Neurite guidance molecules. *Curr Opinion Cell Biol* **1**, 1102-1106
3. Doherty, P., Barton, C.H., Dickson, G , Seaton, P , Rowett, L.H.,

Moore, S.E., Gower, H.J., and Walsh, F.S. (1989) Neuronal process outgrowth of human sensory neurons on monolayers of cells transfected with cDNA for five human NCAM isoforms. *J. Cell Biol.* **109**, 789-798
4. Furley, A.J., Morton, S.B., Manalo, D., Karagogeos, D., Dodd, J., and Jessell, T.M. (1990) The axonal glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite outgrowth-promoting activity. *Cell* **61**, 157-170
5. Bixby, J.L. and Zhang, R. (1990) Purified N-cadherin is a potent substrate for the rapid induction of neurite outgrowth. *J. Cell Biol.* **110**, 1253-1260
6. Letourneau, P.C., Condic, M.L., and Snow, D.M. (1994) Interactions of developing neurons with the extracellular matrix. *J. Neurosci.* **14**, 915-928
7. Smalheiser, N.R., Crain, S.M., and Reid, L.M. (1984) Laminin as a substrate for retinal axons in vitro. *Dev. Brain Res.* **12**, 136-140
8. 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
9. 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
10. 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
11. Rauvala, H. (1989) An 18-kd heparin-binding protein of developing brain that is distinct from fibroblast growth factors. *EMBO J.* **8**, 2933-2941
12. Merenmies, J. and Rauvala, H. (1990) Molecular cloning of the 18-kDa growth-associated protein of developing brain. *J. Biol. Chem.* **265**, 16721-16724
13. 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 a developmentally regulated protein that induces mitogenic and neurite outgrowth activity. *Science* **250**, 1690-1694
14. Muramatsu, T. (1993) Midkine (MK), 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
15. Muramatsu, T. (1994) The midkine family of growth/differentiation factors. *Dev. Growth Differ.* **36**, 1-8
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. Matsumoto, K., Wanaka, A., Takatsuji, K., Muramatsu, H., Muramatsu, T., and Tohyama, M. (1994) A novel family of heparin-binding growth factors, pleiotrophin and midkine, is expressed in the developing rat cerebral cortex. *Dev. Brain Res.* **79**, 229-241
19. 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
20. Kikuchi, S., Muramatsu, H., Muramatsu, T., and Kim, S.U. (1993) Midkine, a novel neurotrophic factor, promotes survival of mesencephalic neurons in culture. *Neurosci. Lett.* **160**, 9-12
21. 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
22. 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
23. O'Reilly, D.R., Miller, L.K., and Luckow, V.A. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*, pp. 150-155, W.H. Freeman and Company, New York
24. Yamada, K., Nakajima, Y., and Natori, S. (1990) Production of recombinant sarcotoxin IA in *Bombyx mori* cells. *Biochem. J.* **272**, 633-636
25. Rauvala, H., Vanhala, A., Castren, E., Nolo, R., Raulo, E., Merenmies, J., and Panula, P. (1994) Expression of HB-GAM (heparin-binding growth-associated molecules) in the pathways of developing axonal processes in vivo and neurite outgrowth in vitro induced by HB-GAM. *Dev. Brain Res.* **79**, 157-176
26. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
27. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354
28. Von Heijne, G. (1983) Patterns of amino acids near signal-sequence cleavage sites. *Eur. J. Biochem.* **133**, 17-21
29. Letourneau, P.C. (1984) in *Molecular Bases of Neuronal Development* (Edelman, G.M., Gall, W.E., and Cowan, W.M., eds.) pp. 269-293, John Wiley & Sons, New York
30. Turnbull, J.E., Fernig, D.G., Ke, Y., Wilkinson, M.C., and Gallagher, J.T. (1992) Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate. *J. Biol. Chem.* **267**, 10337-10341
31. Maccarana, M., Casu, B., and Lindahl, U. (1993) Minimal sequence in heparin/heparan sulfate required for binding of basic fibroblast growth factor. *J. Biol. Chem.* **268**, 23898-23905
32. Ishihara, M. (1994) Structural requirements in heparin for binding and activation of FGF-1 and FGF-4 are different from that for FGF-2. *Glycobiology* **4**, 817-824
33. Lyon, M., Deakin, J.A., Mizuno, K., Nakamura, T., and Gallagher, J.T. (1994) Interaction of hepatocyte growth factor with heparan sulfate. Elucidation of the major heparan sulfate structural determinants. *J. Biol. Chem.* **269**, 11216-11223
34. Ashikari, S., Habuchi, H., and Kimata, K. (1995) Characterization of heparan sulfate oligosaccharides that bind to hepatocyte growth factor. *J. Biol. Chem.* **270**, 29586-29593
35. Raulo, E., Chernousov, M.A., Carey, D.J., Nole, R., and Rauvala, H. (1994) Isolation of a neuronal cell surface receptor of heparin binding growth-associated molecule (HB-GAM). Identification as N-syndecan (syndecan 3). *J. Biol. Chem.* **269**, 12999-13004
36. Silva, M.E. and Dietrich, C.P. (1975) Structure of heparin. Characterization of the products formed from heparin by the action of a heparinase and a heparitinase from *Flavobacterium heparinum*. *J. Biol. Chem.* **250**, 6841-6846
37. Linker, A. and Hovingh, P. (1984) Structural studies on heparin. Tetrasaccharides obtained by heparinase degradation. *Carbohydr. Res.* **127**, 75-94
38. Yanagishita, M. and Hascall, V.C. (1992) Cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* **267**, 9451-9454
39. Lindahl, U., Lidholt, K., Spillmann, D., and Kjellen, L. (1994) More to "heparin" than anticoagulation. *Thromb. Res.* **75**, 1-32