Expression of the Heparin-Binding Growth Factor Midkine in the Cerebrospinal Fluid of Patients with Neurological Disorders

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Abstract

**Objective** This study was to clarify the roles of midkine (MK) in the brain.

**Methods** We determined cerebrospinal fluid MK levels in patients with neurological disorders by enzyme-linked immunoassay and immunostained autopsied brain samples in patients with meningitis.

**Results** MK levels were 0.37±0.21 ng/ml in controls (n=46, mean ± S.D.), 0.67±0.19 ng/ml in patients with cerebral infarction (n=8), 1.78±1.32 ng/ml in patients with meningitis (n=25; ANOVA and post-hoc Fisher’s PLSD test, p<0.0001), 0.31±0.25 ng/ml in patients with human T-lymphotrophic virus type I-associated myelopathy/tropical spastic paralysis (n=29), and 0.42±0.17 ng/ml in patients with amyotrophic lateral sclerosis (n=8). The regression equations were Y=0.005X+0.498 (Y, CSF MK level; X, cell number) and Y=0.007X+0.326 (Y, MK level; X, protein level) for all CSF samples. Autopsy brain samples from patients with meningitis expressed MK weakly in mononuclear cells on immunohistochemical examination. Western blot and polymerase chain reaction analyses showed that leukocytes were MK positive. CSF MK levels were not high in patients with cerebral infarction but were increased in patients with meningitis. CSF MK levels were high in normal controls, compared to those of other cytokines. MK was expressed in choroid plexus of normal brain and released there.

**Conclusion** Our findings suggested that MK may maintain normal adult brain as a neurotrophic factor, and that MK may be released from leukocytes in brain of patients with meningitis as an immunological mediator.

**Key words:** CSF, MK, neurotrophic factor, cytokine, meningitis, cerebral infarction

(DOI: 10.2169/internalmedicine.47.0260)

Introduction

Midkine (MK) is a heparin-binding growth factor with a molecular weight of 13 kDa. It was first isolated as the product of a retinoic acid-responsive gene in an embryonal carcinoma cell differentiation system, and is rich in basic amino acids and cysteine (1-3). Structurally, MK shares approximately 50% sequence identity with pleiotrophin (PTN, also called heparin-binding growth associated molecule, HB-GAM) (4, 5), but it is not related to other growth factors or neurotrophic factors.

Intense MK expression is observed in many tissues during the midgestational period in mice and rats (6-8), while its expression is generally weak in adult tissues. It was previously reported that MK expression is not found in normal adult organs other than kidney (6). MK has various biological activities: it promotes neurite outgrowth (9), fibrinolytic...
activity of endothelial cells (10), survival of embryonic neurons (11), and migration of inflammatory leukocytes (12, 13) and embryonic neurons (14). However, gene knockout mice deficient in MK exhibit only retardation of postnatal development in the hippocampus (15). Following experimental cerebral infarction in rats, MK expression is intensely induced in the surrounding edematous region, though not in the necrotic region in the early stage (16, 17). MK expression is also increased in the hippocampal CA1 subfield following transient forebrain ischemia in rats (18). MK is known to prevent the retinal degeneration induced by exposure to constant light (19).

These observations suggest that MK plays important roles not only in neural development but also in the repair of neural tissue. In addition, histologically-defined tubulointerstitial injury was less severe in MK-deficient (Mdk−/−) mice than in wild-type (Mdk+/+) mice 7 days after renal ischemic perfusion injury due to deficiency of enhancement of migration of inflammatory cells and promotion of inflammation by MK (20).

Recently, serum and urinary MK levels were determined by enzyme-linked immunoassay and found to be increased in patients with various types of carcinoma (21). Unexpectedly, high serum MK levels were found in normal individuals. In the present study, to examine whether or not CSF MK levels are increased in patients with acute stage cerebral infarction and to clarify the roles of MK in the central nervous system, we determined MK levels in cerebrospinal fluid (CSF) in neurologically normal individuals and in patients with cerebral infarction and inflammatory neurological disorders such as meningitis and human T-lymphotropic virus type I (HTLV-I) associated myelopathy/tropical spastic paraparesis (HAM/TSP). HAM/TSP is a chronic, slowly progressive myelitis caused by HTLV-I that presents clinically as paraparesis with neurogenic bladder and mild sensory disturbances, and is characterized by activated T-lymphocyte infiltration of the spinal cord (22).

Materials and Methods

Patients and CSF

The subjects were inpatients at Kagoshima University and related hospitals in Japan. Control CSF samples were obtained during spinal anesthesia from patients undergoing surgery for bone fracture of the leg and without neurological disorders [n=46, 58±22 years (mean ± SD), 17-89 years (25 men and 21 women)]. All of the patients and controls gave informed consent for all procedures. The CSF from patients with acute-stage cerebral infarction other than lacunar stroke was obtained for diagnostic differentiation of brain abscess and tumor (n=8; 6 men and 2 women, 71±5 years, 59-76 years). The level of consciousness of these patients was stupor or less. CSF was collected on the first lumbar puncture soon after admission from patients with meningitis (total number=25; 10 men, 15 women; 45±18 years, 18-81 years; 15 viral, 3 bacterial, 5 tuberculous, and 2 cryptococcal causes), patients with HAM/TSP (n=29; 6 men, 23 women, 60±14 years, 34-80 years) who were ambulatory (walking with or without a cane), and patients with ALS (n=8; 69±9 years, 55-76 years) as a disease control group. ALS patients were diagnosed by neurological status and electromyographic findings. The other patient CSF samples in this study had been collected to determine anti-HTLV-I antibody titer to diagnose HAM/TSP or HTLV-I-related diseases.

Serum MK levels were determined in 19 control subjects without neurological disorders with informed consent (59±6 years, 50-67 years) and in 5 patients with meningitis, whose CSF samples were collected on the same day (50±14 years, 22-62 years).

Nine CSF samples (4 from non-neurological orthopedic patients and 5 from patients with meningitis) were centrifuged at 3,000 rpm for 10 min before freezing. Both the supernatant and corresponding CSF sample before centrifugation were assayed to determine whether or not MK was bound to white blood cells or solubilized in CSF because of basic protein.

Enzyme-linked immunoassay (EIA)

EIA for human MK was performed as described in a previous report (21). Briefly, rabbit anti-human MK antibody was adsorbed onto the wells of microtiter plates (Polysorp plates, Nalge Nunc International KK, Tokyo, Japan), and control human MK samples or serum samples containing peroxidase-labeled chicken anti-human MK antibody were successively added. After incubation for 1 hour, the samples were washed, and color was developed by adding substrate solution (tetramethylbenzidine at 0.5 mg/ml in Dako S 1600, DAKOPATS Inc., Glostrup, Denmark).

Patients with meningitis

Samples from a 9-year-old boy who died of tuberculous meningitis, a 66-year-old man who died of malignant lymphoma complicated by purulent meningitis, and a 59-year-old man who died of acute leukemia complicated by purulent meningitis were subjected to histological study. These patients were autopsied after obtaining consent from their families. Small sections of their brains were immunostained with anti-MK and polyclonal anti-glial fibrillary acidic protein (GFAP) antibodies (DAKO).

Rat brain

Ten-week-old male Wistar rats weighing approximately 300 g were perfused with ca. 50 ml physiological saline containing heparin (2 units/ml) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain was removed, fixed with paraformaldehyde at 4°C overnight, and embedded in paraffin. Then 5 μm-thick sections were cut and subjected to histological and immunohistochemical analyses for detection of MK in normal adult brain.
Histological and immunohistochemical analyses

Sections were stained with hematoxylin and eosin. Immunohistochemistry was performed as previously described (9, 17). Affinity-purified polyclonal rabbit anti-human MK antibody (5 μg/ml) for immunohistochemical staining was prepared and characterized as described previously (17). This anti-MK antibody specifically reacted with MK and not with PTN. To obtain neutralized reagent for use as a negative control, rabbit anti-MK antibody was preincubated with MK, and antigen-antibody complexes were removed by heparin column chromatography (23). Color was developed with DAB.

Western blot and polymerase chain reaction

MK was produced by yeast and purified as previously described (21). White blood cells were isolated on density gradients using Ficoll-Hypaque (Mono-Poly Resolving Medium, specific gravity 1.115, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) from heparinized peripheral blood samples of normal controls following the manufacturer’s instructions, and washed with phosphate-buffered saline. Each 10 μl sample including 3×10⁵ of lymphocytes, 1.2×10⁶ of granulocytes, and 0.96 μg of human MK protein without secretory signal peptide was subjected to electrophoresis, and successive immunoblotting was performed as described previously (17). Reverse transcript-polymerase chain reaction (RT-PCR) of MK messenger RNA (mRNA) of peripheral lymphocytes was performed using a procedure previously described (24).

Statistics

Statistical analyses were performed with a Macintosh computer using StatView version 4.5 (StatView). One-way analysis of variance (ANOVA) was performed, and when a significant F ratio was found, the post-hoc Fisher’s protected least significant differences (PLSD) test was performed on each variable. Student’s t-test was employed in the analysis of serum MK levels. Significance was set at p<0.05.

Results

CSF MK levels were 0.37±0.21 ng/ml in controls (with no significant difference between genders in level), 0.67±0.19 ng/ml in patients with acute-stage cerebral infarction, 1.78±1.32 ng/ml in all patients with meningitis, and 1.21±0.96 ng/ml in patients with tuberculous meningitis, 1.93±1.54 ng/ml in patients with viral meningitis, and 2.01±1.12 ng/ml in patients with bacterial meningitis (Table 1). Statistical analysis was performed for the total meningitis group,

| Table 1. CSF MK Levels in Patients with Tuberculous (TB), Viral, Cryptococcal, and Bacterial Meningitis |
|---|---|---|---|---|---|---|
| patients | sex | Diag | Age (y/o) | MK (ng/ml) | CSF cells /ml | Protein (mg/ml) | Glucose (mg/ml) |
| 1 | man | TB | 68 | 0.894 | 54 | 128 | 60 |
| 2 | man | TB | 57 | 0.746 | 50 | 120 | 58 |
| 3 | man | TB | 37 | 0.464 | 37 | 30 | 57 |
| 4 | woman | TB | 63 | 1.086 | 161 | 99 | 34 |
| 5 | woman | TB | 43 | 2.871 | 212 | 264 | 93 |
| 6 | woman | viral | 61 | 3.667 | 294 | 97 | 21 |
| 7 | woman | viral | 79 | 1.046 | 79 | 157 | 69 |
| 8 | woman | viral | 55 | 1.977 | 168 | 85 | 59 |
| 9 | man | viral | 18 | 0.761 | 157 | 64 | 48 |
| 10 | woman | viral | 21 | 2.878 | 584 | 112 | 58 |
| 11 | woman | viral | 54 | 5.305 | 281 | 125 | 55 |
| 12 | woman | viral | 31 | 3.749 | 188 | 182 | 32 |
| 13 | man | viral | 28 | 0.906 | 49 | 30 | 57 |
| 14 | woman | viral | 24 | 0.766 | 338 | 112 | 49 |
| 15 | woman | viral | 33 | 0.537 | 51 | 108 | 60 |
| 16 | man | viral | 33 | 0.686 | 46 | 84 | 58 |
| 17 | man | viral | 25 | 1.362 | 135 | 103 | 51 |
| 18 | woman | viral | 28 | 1.080 | 349 | 105 | 44 |
| 19 | woman | viral | 45 | 0.509 | 208 | 112 | 65 |
| 20 | man | viral | 42 | 3.770 | 161 | 172 | 40 |
| 21 | man | cryptococcal | 60 | 2.014 | 97 | 159 | 20 |
| 22 | man | cryptococcal | 65 | 1.345 | 150 | 357 | 70 |
| 23 | woman | bacterial | 51 | 1.793 | 645 | 599 | 17 |
| 24 | woman | bacterial | 40 | 3.216 | 40 | 79 | 51 |
| 25 | man | bacterial | 41 | 1.016 | 2048 | 91 | 36 |

average | 45 | 1.78 | 263 | 139 | 50 |
SD | 18 | 1.32 | 404 | 103 | 17 |

Control (mean±SD) | 0.37±0.21 |
Figure 1. Immunohistochemical analysis of MK in the cerebrum of a 9-year-old boy with tuberculous meningitis (a, b, c) and a 66-year-old man (d, e, f) and a 59-year-old man with purulent meningitis (g, h, i). Mononuclear cells infiltrating the cerebral cortex were visualized by Hematoxylin and Eosin staining (a, d, g), and reactive astrocytes were intensely immunostained with anti-glial fibrillary acidic protein antibody (b, e, h). Serial sections revealed that several astrocytes (c; arrow) and the infiltrating mononuclear cells were slightly (c, f, i; *) immunostained with anti-MK antibody. Several nuclei of infiltrating mononuclear cells were intensely immunostained, indicating aggregation of MK in the nuclei. Bar=100 μm.

because there were no significant differences among the meningitis subgroups. CSF MK levels were 0.31±0.25 ng/ml in patients with HAM/TSP and 0.42±0.17 ng/ml in patients with ALS. There were significant differences in MK levels between these groups by ANOVA \( F(4,111)=23.727, p<0.001 \). The post-hoc Fisher’s PLSD test revealed a significant difference between the controls and group of all patients with meningitis \( p<0.0001 \). There were no significant differences between the controls and patients with cerebral infarction, HAM/TSP, or ALS.

The coefficients of correlation \( r \) of CSF MK levels with cell number, protein, and glucose level were 0.611 \( (p<0.0001) \), 0.544 \( (p=0.0001) \), and -0.396 \( (p=0.0008) \), respectively. With CSF cell number denoted X and MK level Y, the regression equation was \( Y=0.005X+0.498 \) \( (n=116, \text{slope, } p<0.0001; \text{constant, } p<0.0001) \). When protein level was denoted X and MK level Y, the regression equation was \( Y=0.007X+0.326 \) \( (p=0.0009, p<0.0001) \). When glucose level was denoted X and MK level Y, the regression equation was \( Y=-0.024X+2.371 \) \( (p=0.0001, p=0.0008) \). CSF MK levels before and after centrifugations were 0.88±1.09 ng/ml and 0.88±1.13 ng/ml, respectively. We therefore concluded that CSF MK was present in supernatant regardless of strong basic protein.

 Serum MK levels were 0.20±0.11 ng/ml \( (n=19) \) in the control subjects and 0.19±0.06 ng/ml \( (n=5) \) in patients with meningitis. There was no significant difference in serum MK level between control subjects and patients with meningitis, as determined by Student’s t-test. The CSF MK level in five patients with meningitis was 1.60±1.0 ng/ml. A significant difference was recognized between CSF and serum levels in normal controls \( p<0.005 \) and patients with meningitis \( p=0.04 \).

The autopsied brain samples from patients with tuberculous and purulent meningitis exhibited meningeal and parenchymal infiltration by mononuclear cells (Fig. 1a, d, g). Immunohistochemically, MK expression was slightly present in mononuclear cells (Fig. 1c, f, i) and in some reactive astrocytes (Fig. 1c). GFAP expression was very intense in areas surrounding mononuclear cell infiltration (Fig. 1b, e, h).

MK was found in choroid plexus of normal rat brain on immunohistochemical analysis (Fig. 2). Western blot analysis showed that lymphocytes and granulocytes contained MK which migrated identically to standard human MK (Fig. 3a), indicating that the MK immunoreactivity in mononuclear cells was indeed due to MK. We were unable to collect a sufficient quantity of monocytes even from ca. 30 ml peripheral blood samples to detect MK in Western blotting.
Discussion

Our initial aim was to determine whether CSF MK levels are high in patients with cerebral infarction, since MK is intensely expressed in the area surrounding experimental cerebral infarcts in rats (17). However, in the present study, slightly increased CSF MK levels were found in patients with meningitis but not in those with cerebral infarction, HAM/TSP, or ALS. MK levels were not particularly high in patients with meningitis because CSF cell numbers or protein levels were high in only a few of the patients examined. MK levels soon decreased with improvement of meningitis on repeated determination in several patients. The regression equations indicated that the MK level increased with increases in cell number and protein level. The results of Western blot analysis indicated that lymphocytes contained more MK protein than granulocytes. Although MK concentration is thought to be increased in disorders that increase CSF lymphocytes such as viral meningitis, we could not determine in which, viral or bacterial meningitis, CSF MK was higher, due to the small number of patients in the present study. HAM/TSP is a chronic progressive myelitis, associated with milder inflammation than meningitis (22). The present findings are reminiscent of the increased MK levels in synovial fluid of patients with rheumatoid arthritis (13) and they suggest that MK functions as an inflammatory cytokine in the central nervous system. Neutrophilic factors such as brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3/4, and nerve growth factor (NGF) are known to be immunological cytokines, produced by lymphocytes and uptaken by lymphocytes in autocrine mode (25), similar to the present findings for MK.

Normal or non-meningitic CSF cytokine levels have been reported to be as follows: interferon (INF)-γ, 4.8±0.9 pg/ml (n=11) (26); TNF-α, less than 10 pg/ml (27); interleukin (IL)-1β, non-detectable (28); IL-6, 28±20 pg/ml (n=11) (26); IL-8, 47±64 pg/ml (n=25) (29); IL-10, less than 10 pg/ml (27); and IL-18, 0.10±0.04 pg/ml (n=30) (30). Levels of these cytokine are increased in patients with meningitis, as follows: INF-γ, 36±38 pg/ml (viral meningitis, n=79) (26), 13.85±5.51 pg/ml (bacterial meningitis, n=12), and 36.28±19.45 pg/ml (viral meningitis, n=12) (30); TNF-α, 1.372±2,835 pg/ml (bacterial meningitis, n=12) (31); IL-1β, 635±1,553 pg/ml (bacterial meningitis, n=12) (31) and 1,750 pg/ml (median value, range <11-4,000, bacterial meningitis, n=35) (27); IL-10, 701 pg/ml (median value, range <11-4,000, bacterial meningitis, n=35) (27); IL-18, 75.51±23.25 pg/ml (bacterial meningitis, n=6) and 3.23±1.37 pg/ml (viral meningitis, n=6) (30). The present findings suggest that, compared to those reported above, CSF MK levels were increased in patients with meningitis, though control CSF level was 0.37±0.21 ng/ml, and rather high compared to those of the other cytokines. Although quantitative determination generally depends on the precision of the assay system used for EIA, the EIA system we used has been tested well for precision and reproducibility (21). Concerning the high MK levels in control CSF, the present finding that choroid plexus epithelium was immunoreactive to MK suggested that the cells producing MK might be those of choroid plexus in normal brain. Syndecan-1 and -3 (33), receptor-like protein-tyrosine phosphatase ζ (14), and LDL receptor-related protein (LRP) (34) have recently been reported as MK receptors. LRP is a typical endocytotic receptor. Binding of MK to LRP resulted in internalization by endocytosis, and MK emerged in cytoplasm (10, 35). The finding of anti-MK-positive cells probably demonstrated that, once released, MK was taken up.
were stained. RT-PCR showed that mRNA was present in phocytes; however, we could not confirm that monocytes ing by this antibody. Therefore, large numbers of granulocytes were stained by anti-MK antibody in our experiments. We examined histochemical study, lymphocytes were always immunopositive to anti-MK antibody in our experiments. Our findings suggest that high levels of MK were derived from infiltrating leukocytes in meningitis.

It has been shown that astrocytes produce MK in the areas surrounding infarcts or ischemia in the early but not the chronic stage (16-18, 36, 37). In the autopsied cerebral immunostained sections we examined, MK was expressed only slightly and in only some of the astrocytes adjacent to the infiltrating mononuclear cells, perhaps because examination was performed in the chronic stage.

Our findings suggest that MK, a neurotrophic factor, is always expressed in normal adult brain for neuronal maintenance, and that it plays a role as an inflammatory cytokine such as BDNF, NT-3/4, and NGF in states of inflammation. The roles of MK as an immune mediator remain to be clarified.

References


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