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# Enzyme-Linked Immunoassay for Midkine, and Its Application to Evaluation of Midkine Levels in Developing Mouse Brain and Sera from Patients with Hepatocellular Carcinomas[1]

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**Midkine (MK) is a growth factor that promotes neurite outgrowth and survival of neurons, and enhances the plasminogen activator in endothelial cells. A highly sensitive enzyme-linked immunoassay for MK was developed, involving affinity-purified anti-MK antibodies, their biotinylated form, and avidin-β-galactosidase. The amount of bound avidin-β-galactosidase was determined using a fluorogenic substrate, 4-methylumbelliferyl-β-D-galactoside. This method allowed the detection of human and mouse MK in the range of 50 pg-10 ng. Pleiotrophin, which is related to MK in its amino acid sequence, did not show any cross reactivity. Employing this method, the MK levels in the developing mouse brain were determined. The MK level was 2 μg/g of wet tissue on the 12th day of gestation, and then steadily decreased during embryogenesis and postnatal development to 30 ng/g two months after birth. The assay method can also be applied to serum samples. Although the MK levels in the sera of normal human subjects were low or undetectable, 0.6-8 ng/ml of MK was detected in samples in the majority of cases of hepatocellular carcinomas.**

**Key words: brain development, enzyme immunoassay, hepatocellular carcinoma, midkine.**

Midkine (MK) is a heparin-binding growth factor of a molecular weight of 13 kDa, rich in basic amino acids and cysteine (*1, 2*). MK is structurally unrelated to fibroblast growth factors, but exhibits 45% amino acid sequence indentity with pleiotrophin (PTN, also called HB-GAM) (*3, 4*); MK and PTN form a unique family of growth factors called the MK family (*5, 6*). MK promotes the neurite outgrowth and survival of embryonic neurons (*7-9*), and enhances the plasminogen activator level in aortic endothelial cells (*10*).

MK is strongly expressed in various tissues during the midgestation period of mouse embryogenesis, and is considered to be involved in the regulation of organogenesis (*11-13*). Furthermore, MK expression has been found to be increased in the majority of cases of various human carcinomas: Wilms' tumors and, hepatocellular, lung, breast gastric, colon, and pancreatic carcinomas (*14-17*). In the case of Wilms' tumors, all six surgically-removed specimens strongly expressed MK mRNA, while a non-cancerous kidney specimen only weakly expressed it (*14*). The strong expression of MK mRNA was correlated with a worse prognosis in patients with neuroblastomas, whereas the strong expression of PTN was correlated with an improved prognosis in patients with the same disease (*18*). MK was also detected in senile plaques of all cases of Alzheimer's disease, but not in normal portions of the brain (*19*). MK is detectable in the edematous region in the early stages of experimental brain infarction, suggesting that it is involved in the repair of damaged tissues (*20*). Indeed, MK prevents damage to outer photoreceptor segments caused by exposure to constant light (*21*). These physiological activities of MK, as well as possible involvement of MK in pathological processes, made it necessary to develop a highly sensitive assay system for MK. This study describes an enzyme-linked immunoassay for MK. The utility of the assay was evaluated by measuring the MK levels in developing mouse brain and sera from patients with hepatocellular carcinomas.

## MATERIALS AND METHODS

*Materials*—Recombinant mouse MK produced by L cells (*7*), and chemically synthesized human MK (*22*) were prepared and purified, as described in the respective references. Rabbit antibodies were raised against the MK preparations mentioned above, and were affinity purified as described previously (*8*). As the affinity ligand for the purification of anti-mouse MK, *Escherichia coli*-produced MK (*23*) was used, while maltose-binding protein-MK

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Abbreviations: MBP-MK, maltose-binding protein midkine; MK, midkine; PBS(−), Dulbecco's phosphate-buffered saline, without $Ca^{2+}$ and $Mg^{2+}$; PTN, pleiotrophin; Washing buffer, 100 mM Tris-HCl buffer, pH 7.5, containing 0.4 M NaCl, 0.1% bovine serum albumin, 0.1% $NaN_3$, and 1 mM $MgCl_2$.

fusion protein (MBP-MK) was used for the purification of anti-human MK. MBP-MK was produced in *E. coli*, transformed with an expression vector in which a cDNA fragment, corresponding to 20-143 amino acids of human MK, was placed in frame following a maltose binding protein cDNA sequence, and purified as described (*23*). Biotinylated antibodies were prepared using NHS-LC-Biotin (Pierce) as recommended by the manufacturer. Streptoavidin-$\beta$-galactosidase and 4-methylumbelliferyl-$\beta$-D-galactoside were purchased from Boehringer Mannheim and Sigma, respectively. PTN was purchased from R & D Systems.

*Samples*—Brain tissue (0.06-0.54 g) from embryonic, postnatal or adult ICR mice was homogenized for 5 min at 4°C, in 0.6-5.4 ml of 1 M NaCl in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 105,000×$g$ at 4°C for 60 min. The supernatant was diluted with the washing buffer [100 mM Tris-HCl buffer (pH 7.5), 0.4 M NaCl, 0.1% bovine serum albumin, 0.1% $NaN_3$, and 1 mM $MgCl_2$], without NaCl, and used for the assay.

Blood taken from patients with hepatocellular carcinomas and from normal human subjects was allowed to clot by leaving it at room temperature overnight, and the resulting serum was used for the assay after dilution two-times with Dulbecco's phosphate-buffered saline, without $Ca^{2+}$ and $Mg^{2+}$ [PBS(−)].

*Heparin Affinity Chromatography*—Samples to be assayed (100 $\mu$l-1 ml), prepared as described above, were applied to a column of heparin-Sepharose (0.2 ml) (Pharmacia), which had been equilibrated with 0.2 M NaCl in 20 mM Tris-HCl buffer (pH 7.5). After washing with 0.5 M NaCl in 20 mM Tris-HCl buffer (pH 7.5), heparin binding proteins, including MK, were eluted with 1.5 M NaCl in the buffer.

*Assay Method*—Affinity-purified anti-MK antibodies (1 $\mu$g) in 50 $\mu$l of 50 mM Tris-HCl buffer (pH 8.0) were added to a well in a microtiter plate (Falcon 3915). After incubation at room temperature for 3 h, the antibody solution was removed and the well was washed with PBS(−). After blocking with the washing buffer for 1 h, 50 $\mu$l of a sample solution was added, and the plate was incubated at room temperature for 3 h. The sample was removed and the well was washed with the washing buffer. Biotinylated anti-MK antibodies (0.7 ng in 50 $\mu$l of washing buffer) were added, and allowed to react at 4°C overnight. Then 5 mU of streptoavidin-$\beta$-D-galactosidase conjugate in 50 $\mu$l of washing buffer was added, and the plate was incubated at room temperature for 1 h. After the solution had been removed and the well washed with the washing buffer, 5 $\mu$g of 4-methylumbelliferyl-$\beta$-D-galactoside in 50 $\mu$l of washing buffer was added, followed by incubation at room temperature for 4 h. The enzyme reaction was stopped by adding 200 $\mu$l of 0.1 M glycine-NaOH buffer (pH 10.3). Then the amount of 4-methylumbelliferon released was measured with a microplate fluorescence reader (Cytofluor II, Biosearch), with excitation and emission wavelengths at 360 and 460 nm, respectively.

## RESULTS AND DISCUSSION

*Enzyme-Linked Immunoassay for MK*—An enzyme-linked immunoassay for MK was developed as follows. Affinity-purified anti-MK antibodies were absorbed to the well of a microtiter plate, and then a sample solution containing MK was added to the well. Then biotinylated anti-MK antibodies were added, and the biotin-labeled antibody absorbed was quantified enzymatically, with the aid of avidin-labeled $\beta$-galactosidase and its fluorogenic substrate.

This method allowed estimation of mouse MK (Fig. 1) and human MK (Fig. 2) in the range of 50 pg-10 ng/ml (2.5-500 pg in absolute amounts). The intraassay coefficients of variation (CV) were 9.5 and 2.5% for 100 and 800 pg/ml of mouse MK, respectively. The coefficient of correlation ($r^2$) was 0.966 for mouse MK and 0.980 for human MK.

The anti-MK antibodies are highly specific and do not react with PTN, even though they exhibit 45% sequence identity (*8*). Indeed, 1 $\mu$g of human PTN showed no reactivity at all on the assaying of human MK. In addition, on the assaying of mouse MK, human MK showed only about 2.0% reactivity, compared to the dose of MK giving a fluorescence intensity of 0.05 (Fig. 3). Therefore, the assay is sensitive, reproducible and highly specific for

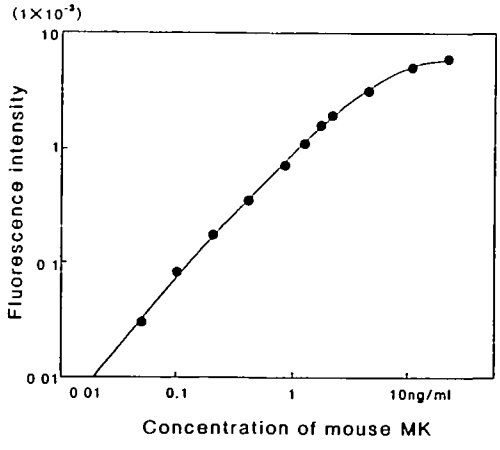


Fig. 1. **Enzyme immunoassaying of mouse MK.** The fluorescence intensity in the absence of MK, usually 200-300, was subtracted from the observed value, and then plotted.

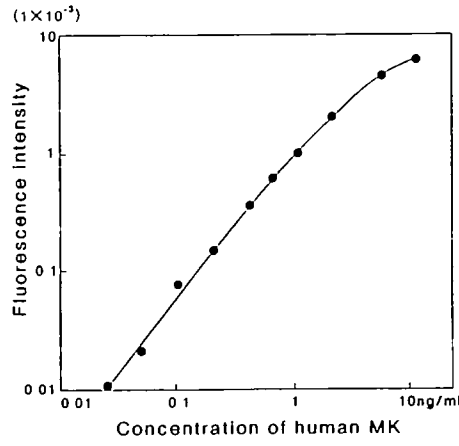


Fig. 2. **Enzyme immunoassaying of human MK.** The fluorescence intensity in the absence of MK, usually 200-300, was subtracted from the observed value, and then plotted.

mouse and human MK.

*Determination of MK Levels in the Developing Mouse Brain*—Northern blot analysis, Western blot analysis, *in situ* hybridization and immunohistochemical staining have shown that MK is abundantly expressed in the brain of midgestation embryos, but is scarcely detectable in the brain of adult mouse, rat, and man (*8, 11, 19, 20, 24*). However, the actual levels of MK in the brain have not been determined.

We extracted MK from mouse brains at various developmental stages and the amounts of MK in the extracts were determined by enzyme-linked immunoassaying. The amount of MK in the brain of a 12-day-old embryo was 2 μg/g of brain, and then the level gradually decreased during development (Fig. 4). Two months after birth, only 30 ng/g of MK was detected. In order to evaluate the accuracy, extracts of 12 and 15-day-old embryonic mouse brains, and ones of 1- and 2-month-old mouse brains were applied to a heparin-Sepharose column. Determination of the MK levels in the eluates confirmed the above-mentioned MK levels in all cases within an error of ±10%. Thus, MK immunoreactivity measured in the assay is not due to unrelated material in an extract, and the assay is not affected by possible interfering substances in the extract.

The amount of MK in the 12-day-old embryonic brain, 2 μg/g, was extremely high. MK is known to act on embryonic neurons in two different ways. Neurite-promoting activity is seen when 5–10 μg/ml of MK is coated on the surface of a dish (*7, 8*), and survival-promoting activity is seen on the addition of 10 ng/ml of soluble MK to the medium (*9*). The presence of a large amount of MK in the embryonic brain is consistent with the view that MK is physiologically active not only as to survival-promoting activity, but also neurite-promoting activity. Considering that MK is mostly on the cell surface and in the extracellular space in the embryonic brain (*8*), the extracellular concentration of MK in the 12-day-old embryonic brain can easily reach the level of 5–10 μg/ml, which is used for neurite outgrowth assays.

Even in the adult brain, a small amount of MK was detected. We have not yet localized the site of MK accumulation in the adult brain. It is possible that some MK is present in blood vessels, since heparin administration is known to increase MK in the blood (*25*). However, we should not overlook the possibility that a small amount of MK, which could have escaped immunohistochemical detection, is present in neurons of the adult brain, and has certain functions.

*Determination of MK Levels in Sera of Normal Human Subjects and Patients with Hepatocellular Carcinomas*—With respect to clinical applications of the enzyme-linked immunoassay for MK, the most valuable is the determination of MK levels in sera. Thus, in this study, we examined the effect of serum on the determination. Although the response curve did not change upon the addition of serum (data not shown), the background level decreased with the addition of serum (Table I). This is probably due to a decrease in the non-specific adhesion of reagents by coating the microtiter plate with serum proteins. Thus, we used normal human serum, which was passed through a heparin-Sepharose column to remove MK if present, as a control to determine the MK levels in serum samples. Using this control, the MK levels in human sera could be measured using sera diluted twice with PBS(−). The MK levels in sera of normal human subjects were either undetectable or less than 0.6 ng/ml in the 67 cases examined (Fig. 5). However, in more than half the hepatocellular carcinoma patients, MK was detectable in sera in the range of 0.6–8 ng/ml. In all positive cases, heparin affinity chromatography confirmed that the antigenic material showed the expected behavior of MK. Although the majority of hepatocellular carcinomas express MK, the rest do not (*14, 17*). Therefore, an obvious reason for a negative MK level

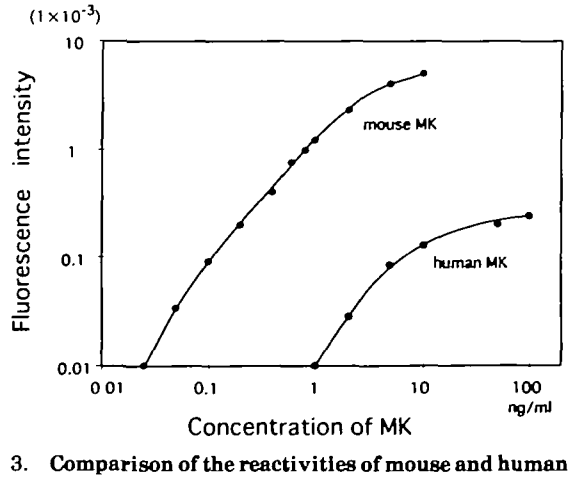


Fig. 3. **Comparison of the reactivities of mouse and human MK on enzyme immunoassaying of mouse MK.**

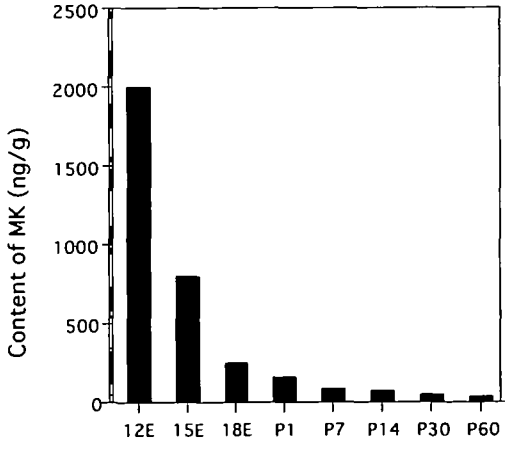


Fig. 4. **Assaying of MK levels in developing mouse brains.** The results are expressed as the amount of MK in 1 g of brain. E, embryonic days; P, postnatal days.

TABLE I. **Effect of serum on the background level in the MK assay.**

| | Relative fluorescence intensity without MK |
|---|---|
| Washing buffer | 1.00 |
| Fetal calf serum+PBS(−) (1 : 1) | 0.65 |
| Normal calf serum+PBS(−) (1 : 1) | 0.60 |
| Normal human serum[a]+PBS(−) (1 : 1) | 0.67 |
| Normal human serum[b]+PBS(−) (1 : 1) | 0.64 |

[a]Before application to the heparin-Sepharose column. [b]After application to the heparin-Sepharose column.

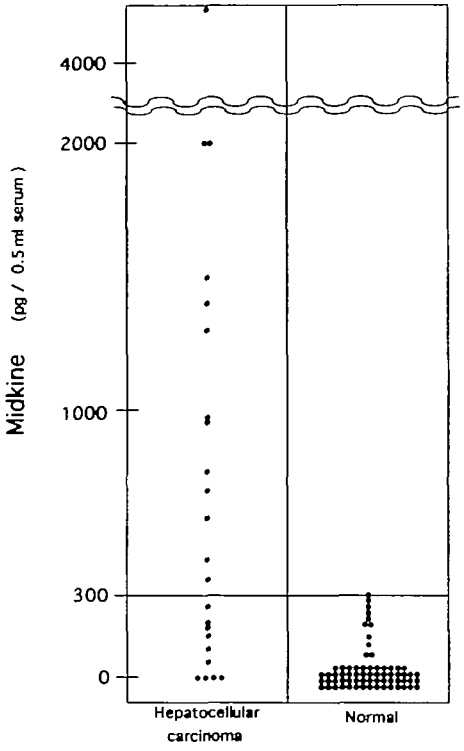


Fig. 5. **Comparison of the MK levels in sera of patients with hepatocellular carcinomas and normal human subjects.** For the assays, sera were diluted with an equal volume of PBS(−). The results are expressed in ng of MK in 1 ml of sample solution, or 0.5 ml of serum.

in serum is the lack of MK expression in the carcinoma. Also we made preliminary MK level measurements in sera of chronic liver disease cases, and found some positive ones. Thus, further studies are required to determine whether the assaying of MK is helpful in the diagnosis or follow-up of hepatocellular carcinomas.

In any event, the results of the present investigation clearly indicated that this assay method for MK is appropriate for MK determination in sera. It will be especially interesting to examine the MK levels in sera of patients with various carcinomas other than hepatocellular ones. We have already observed that the serum MK levels were increased in certain patients with colon carcinomas, and that the level might be correlated with the tumor grade (unpublished results).

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