THE SERUM LEVEL OF MIDKINE, A HEPARIN-BINDING GROWTH FACTOR, AS A TUMOR MARKER

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ABSTRACT

Midkine (MK) is a heparin-binding growth factor distinct from fibroblast growth factors. Serum levels of MK were determined by enzyme-linked immunoassay using affinity-purified anti-human MK antibody. Elevated levels of MK were frequently observed in sera from patients with various carcinomas including lung carcinoma, bile duct carcinoma, colon carcinoma and esophageal carcinoma. Most patients with lung carcinoma showed high MK serum values. In colorectal carcinoma, some correlation was observed between high MK value and tumor invasion. Surgical removal of carcinomas invariably resulted in decreases in the MK level. Determination of serum MK may be useful as an aid in initial screening of certain carcinomas, such as lung carcinoma.

Growth factors and members of the signal transduction system are frequently overexpressed in tumors, contributing to their malignant phenotypes (1, 4, 8). Therefore, growth factors and the signal transducers may be used as tumor markers to detect and follow the clinical course of malignancy.

We evaluated the possible utility of midkine (MK) serum levels as a tumor marker. MK is a heparin-binding growth factor with a molecular weight of 13 kDa, structurally unrelated to fibroblast growth factors (FGFs) (11, 27), and together with pleiotrophin (PTN)/heparin-binding growth associated molecule (HB-GAM) forms a new family of growth factors (14, 15, 23). MK enhances neurite outgrowth and survival of embryonic neurons (16, 20, 21) and enhances plasminogen activator activity of endothelial cells (13). Although MK is only weakly expressed in most normal adult tissues, strong MK expression was found in embryo-

nic and damaged tissues (10, 17, 30) and in various carcinomas (3, 6, 7, 24, 25). MK appears to be involved in formation and repair of tissues; indeed, MK administration is known to prevent damage of photoreceptor outer segments caused by exposure to constant light (29). Increased MK expression in carcinomas may reflect aberrant tissue formation and repair in carcinogenesis. We have developed a highly sensitive enzyme immunoassay for MK (22). Using this assay, normal human serum was shown to contain undetectable or low levels of MK, whereas in the majority of cases of hepatocellular carcinoma the serum MK level was markedly increased (22). Therefore, we examined the MK level in sera of various cancer patients to determine whether the MK level is increased in other malignancies.

MATERIALS AND METHODS

Enzyme-Linked Immunoassay

Affinity-purified rabbit anti-human MK antibody was prepared as described previously (22). Enzyme-linked immunoassay for MK was per-

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Table 1 Serum MK Levels in Patients with Primary Tumors

Disease	Case number	Age	Sex	Degree of differentiation	Lymph node metastasis	MK (pg/0.5 ml)
Esophageal carcinoma ¹	1	62	M	Moderate	—	0
	2	48	M	Inoperable	—	0
	3	47	M	Well	+	2,500
	4	70	M	---	—	65
	5	50	M	Well	+	325
	6	73	M	Well	—	23
	7	68	M	Moderate	—	37
	8	67	F	Well	—	92
	9	65	M	Well	+	160
	10	61	M	Moderate	—	5,000
	11	55	M	Moderate	—	215
	12	57	F	Poor	+	132
	13	58	M	Moderate	—	530
	14 [#]	61	M	Poor	+	1,579
Gastric carcinoma ²	15	67	M	Inoperable	—	225
	16	65	M	Well	—	1,310
	17	37	F	---	—	135
	18	42	M	---	—	63
	19	76	M	Well	+	69
	20	74	M	Well to moderate	+	128
Duodenal ampullary carcinoma ³	21	66	M	---	+	83
Carcinoma of the jejunum ⁴	22	64	F	---	+	0
Colorectal carcinoma ⁵	23	66	M	Moderate	+	610
	24	67	F	Moderate	+	2,600
	25	77	M	Well	—	0
	26	68	M	Moderate	—	400
	27	81	F	Well	—	175
	28	84	F	Well	—	108
	29	71	M	Well	—	281
	30	61	F	Moderate	+	299
	31	78	M	Moderate	+	305
	32	48	M	Moderate	—	54
	33	76	F	Moderate	+	145
	34 [*]	34	F	---	---	981
Hepatocellular carcinoma	35	69	M	Moderate	---	620
	36	78	M	Moderate	---	2,500
	37	61	F	Moderate	---	8,000
	38	52	M	Well to moderate	---	7,400
	39	64	M	Moderate	---	2,891
Bile duct carcinoma ⁶	40	57	M	---	—	0
	41	63	F	Poor to moderate	+	480
	42	72	F	Moderate	+	210
	43	58	M	Poor to moderate	—	1,290
	44	69	M	Moderate	—	207
	45	47	M	Poor to moderate	—	206
	46	74	M	Moderate	—	389
Gallbladder carcinoma	47	70	F	Inoperable	—	0
Pancreatic carcinoma	48	68	F	Inoperable	—	71
	49 [§]	68	F	Well	—	430
Thyroid carcinoma	50	55	F	---	—	209
	51	59	F	---	—	205
Lung carcinoma	52	69	M	Poor	—	450
	53	66	M	Moderate	+	850
	54	59	M	Moderate	—	455
	55	74	F	Well	—	720
	56	44	M	---	+	1,450
	57	67	M	Moderate	+	210
	58	53	M	Moderate	+	1,713
Breast carcinoma	59 [†]	70	F	---	---	1,250

formed by a modification of the method of Muramatsu *et al.* (22). Affinity-purified anti-human MK antibodies were absorbed onto the wells of a microtiter plate (Falcon 3915). After incubation at room temperature for 3 h, the wells were blocked with washing buffer (100 mM Tris-HCl buffer, pH 7.5, containing 0.4 M NaCl, 0.1% NaN₃, 1 mM MgCl₂ and 0.1% bovine serum albumin) for 1 h. Then, sera taken from patients were added to the wells after twofold dilution with Dulbecco's phosphate-buffered saline without Ca²⁺ or Mg²⁺ [PBS(-)]. The plates were incubated at room temperature for 3 h, then biotinylated anti-MK antibodies were added and allowed to react at 4°C overnight. Then, 5 mU of streptavidin- β -D-galactosidase conjugate was added, and incubated at room temperature for 1 h. After 4-methylumbelliferyl- β -D galactoside was added, and after incubation at room temperature for 4 h, the enzyme reaction was stopped by addition of 0.1 M glycine-NaOH (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. As a standard, we used chemically synthesized human MK (Peptide Institute), which was dissolved in 1:1 mixed solution of PBS(-) and normal human serum passed through a heparin-Sepharose column.

Sera from Patients

Blood taken from patients was allowed to clot by leaving it at room temperature overnight, and the resulting serum was used for the assay after twofold dilution with PBS(-).

RESULTS

Serum MK Levels in Patients with Various Carcinomas

We determined MK levels in sera from 59 patients with various carcinomas (Table 1) using a highly sensitive enzyme immunoassay. In 67 normal human sera simultaneously determined, MK levels were less than 300 pg/0.5 ml, as reported previously (22). Thus, we regarded MK values of more

than 300 pg/0.5 ml as significantly high.

In all 5 cases of hepatocellular carcinomas, high levels of MK were detected in the sera, confirming the findings described in our previous report (22) (Table 1). High levels of serum MK were found in 6 of 7 cases of lung carcinoma, 3 of 7 cases of bile duct carcinoma, 5 of 12 cases of colon carcinoma, and 5 of 14 cases of esophageal carcinoma were also positive (Table 1). One of 6 cases of gastric carcinoma, 2 cases of pancreatic carcinoma and one case of breast carcinoma were also positive (Table 1). Thus, in many carcinomas, the serum MK level was elevated.

In colorectal carcinoma, high MK values were encountered in 3 of 5 cases in which lymph node metastasis was found, while it was encountered only in 1 of 6 cases in which there was no lymph node metastasis (Table 1). Furthermore, high MK values were found in all 3 cases in which tumors invaded the subserosal layer (cases 23, 24 and 26). It should be noted that there was no lymph node metastasis in case 26 even though the MK value was high. Although more extensive studies are needed, the results obtained to date suggest that MK serum levels are correlated with tumor invasion to a certain extent in colorectal carcinoma.

In other cases of carcinomas, we could not detect significant correlation between pathological features and serum MK levels.

Serum MK Levels before and after Surgery

We examined serum MK levels before and after surgery in 8 cases of carcinoma. In all 8 cases, serum MK levels decreased after surgery, including two cases of gastric carcinoma in which the pre-operative value of serum MK was low (Fig. 1). The decrease was not marked in patients with gastric carcinoma, one patient with esophageal carcinoma, and in one patient of colorectal carcinoma (Fig. 1). In other cases, the decreases were marked. It should be noted that the esophageal carcinoma patient also had untreated oral carcinoma.

Serum MK Levels in Hepatic Diseases

Since high levels of MK were detected in sera of

¹Squamous cell carcinoma, except for Case 4 which was adenosquamous cell carcinoma. ²Adenocarcinoma, except for Cases 17 and 18 which were signet ring carcinoma. ³Adenocarcinoma. ⁴Tubular adenocarcinoma. ⁵Adenocarcinoma. ⁶Adenocarcinoma (Cases 40-43) or tubular adenocarcinoma (Cases 44-46). ⁷Double cancer. ^{*}Focal carcinoma in tubular adenoma. ⁸Tubular adenocarcinoma. [†]Invasive ductal carcinoma. --- not defined; +, present; -, absent

Fig. 1 Serum MK levels of patients before (■) and after (▨) surgery. C, carcinoma; 1, case number 39; 2, case number 58; 3, case number 14; 4, case number 59; 5, case number 34; 6, case number 31; 7, case number 20; 8, case number 19

many patients with hepatocellular carcinoma, we examined sera of patients with hepatitis to determine whether non-malignant hepatic diseases also caused elevated serum MK levels. In some cases of hepatitis caused by acute A, B virus or C virus and non B, non C hepatitis, serum MK levels were elevated (Fig. 2). This was also the case of liver cirrhosis. As above, in certain non-cancerous hepatic diseases, MK serum levels increased. However, the frequency of the elevation was low, and the possibility was not excluded that in some of the patients undetectable carcinomas were present.

DISCUSSION

MK expression is significantly associated with tumorigenesis. Firstly, MK is overexpressed in a variety of human tumors such as hepatocellular (28), esophageal (3), colon (3), pancreatic (3), lung (6), breast (7), stomach (3) and bladder (25) carcinomas. In more than 80% of cases of the above carcinomas, MK overexpression is evident. In neu-

roblastoma (24) and bladder carcinoma (25), strong MK expression is correlated with poor prognosis. Secondly, MK is overexpressed in all cases of Wilms' tumor specimens (28), and WT1, a tumor suppressor gene whose loss is associated with Wilms' tumor, has been found to suppress human MK promoter function (2). Finally, transfection of murine MK cDNA and its overexpression lead to oncogenic transformation of the recipient NIH3T3 cells (9). Therefore, overexpression of MK in human tumors appears to be correlated with their malignant phenotype. The general expression of MK in human tumors and its probable role in malignant phenotype make MK an excellent candidate as a tumor marker.

Indeed, we found that serum MK levels are increased in many carcinomas, not only in hepatocellular carcinoma but also in lung, bile duct, colon and esophageal carcinomas and others. This is in agreement with the elevated MK mRNA levels observed in many human carcinomas (3, 6, 7, 28). Due to the low or non-detectable levels of MK in

Fig. 2 Serum MK levels of patients with various hepatic diseases. AH, acute hepatitis; CH(B), chronic hepatitis caused by hepatitis virus B; CH(C), chronic hepatitis caused by hepatitis virus C; CH(B + C), chronic hepatitis caused by hepatitis virus B and C; CH(NBNC), chronic non B non C hepatitis

sera of large numbers on normal human subjects, serum MK levels may be a useful marker in initial screening for cancer. It is especially interesting that high MK levels were encountered in most cases of lung carcinoma. Furthermore, in colorectal adenocarcinoma, high levels of serum MK may be associated with invasive characteristics. In this respect, it is proper to mention that both PTN and MK have been shown to exhibit angiogenic activity (5, 25, 26), and destruction of PTN mRNA by ribozyme

resulted in reduction of invasive characteristics of melanoma (5) and choriocarcinoma (26).

Another interesting aspect regarding MK and malignancy is that a truncated form of MK mRNA, which encodes MK devoid of the N-terminal domain has been so far detected only in tumor specimens but not in adjacent non-cancerous tissues (12, 18, 19). Although we do not know the physiological significance of the expression of the truncated mRNA, this can be used to monitor the

localization of micrometastases in lymph nodes. Both the serum MK level and the truncated MK mRNA in cancerous tissue may be used as tumor markers.

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