Frequent Expression of Midkine Gene in Esophageal Cancer Suggests a Potential Usage of Its Promoter for Suicide Gene Therapy

Motohiro Miyauchi, Hideaki Shimada, Kenji Kadomatsu, Takashi Muramatsu, Shuichiro Matsubara, Shinya Ikematsu, Keizo Takenaga, Takehideo Asano, Takenori Ochiai, Shigeru Sakiyama and Masatoshi Tagawa

Divisions of 1Pathology and 1Chemotherapy, 2Chiba Cancer Center Research Institute, 666-2 Nitona, Chuo-ka, Chiba 260-8717, 2Department of Surgery (II), Chiba University School of Medicine, 1-8-1 Inohana, Chuo-ka, Chiba 260-8670, 3Department of Biochemistry, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, 4Department of Biochemistry, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520 and 5Meiji Cell Technology Center, 540 Naruda, Odawara 250-0862

We have examined the expression of midkine (MK), a neurotrophic factor with heparin-binding activity, in human esophageal cancer cells. Seven esophageal cell lines tested expressed the transcript and 8 out of 14 human esophageal tumor specimens were positively stained with anti-MK antibody, while surrounding normal esophageal tissues in these specimens were not stained. The 5′-flanking, 2.3 kb genomic region of the MK gene was shown to drive the transcription of a reporter gene in the esophageal cell lines in a cis acting manner. Forced expression in esophageal cancer cells of herpes simplex virus-thymidine kinase gene mediated by the flanking region of the MK gene conferred sensitivity to a prodrug, ganciclovir. The 5′-upstream region of the MK gene thus possesses putative promoter activity which can be used for suicide gene-based gene therapy for esophageal cancer.

Key words: Midkine — Suicide gene — HSV-TK — Esophageal cancer — Gene therapy

Midkine (MK) is a heparin-binding growth factor and its expression is conserved across species and is developmentally regulated.1-3 It promotes neurite outgrowth and has a mitogenic activity towards fibroblasts and neuroectoderm cells.4,5 The biological function of MK during tumorigenesis is not clearly understood, though MK expression is elevated in various types of human cancer such as Wilms’ tumor,6 breast cancer,7 and neuroblastoma.8 Recent studies have shown that MK plays a possible role in enhancing angiogenesis9 and that overexpression of the MK gene is a poor prognostic factor for patients with bladder carcinoma.10 In normal adult tissues, MK is expressed solely in the kidney, lung and small intestine.6 The limited expression in normal tissues implies a possible use of the promoter, which preferentially functions in tumor cells, for therapeutic strategies based on tumor-specific gene expression.

Esophageal cancer remains one of the most intractable tumors due to the higher incidence in aged patients and its rapid infiltration into neighboring tissues despite multimodal approaches.11 Novel procedures, therefore, should be introduced to improve the prognosis, and gene therapy is a possible therapeutic approach.

Increased MK expression in human gastrointestinal tumors was reported,12 but the expression in esophageal cancer has not been extensively studied. In this study, we examined the expression of the MK gene in human esophageal cancer and found that it was detectable solely in the tumors. We then tested whether the 5′-upstream region of the MK gene could be used as a promoter to generate transcriptional activation in esophageal tumors. For that purpose, the expressions of a reporter gene and the herpes simplex virus-thymidine kinase (HSV-TK) gene were investigated in human esophageal cancer cell lines, using the upstream region of the MK gene.

MATERIALS AND METHODS

Cells Human esophageal cell lines were cultured in RPMI1640 medium supplemented with 10% fetal calf serum. T.Tn cells were from Japanese Cancer Research Resources Bank and ECGI10 cells from Riken Cell Bank (Tsukuba). TE1, TE2, TE10, TE11 and TE13 cells were kindly provided by Dr. T. Nishihara (Tohoku University, Sendai). A human melanoma line, A875 from Dr. A. Nakagawara (Chiba Cancer Center, Chiba) and a human foreskin fibroblast line, HFF from Dr. T. Yamakawa (Tokyo Metropolitan Institute of Gerontology, Tokyo) were
Expression of MK protein was analyzed with anti-MK antibody. TNM was based on the classification by the International Union Against Cancer.

Expression of MK protein was analyzed with anti-MK antibody. TNM was based on the classification by the International Union Against Cancer.

**Table I. Expression of MK Protein in Human Esophageal Tumor Specimens and Clinicopathological Data of the Patients**

| Patient No. | Age/Gender | MK expression | TNM | Stage (Pathology) | Differentiation |
|-------------|------------|---------------|-----|------------------|----------------|
| 1           | 60/M       | T3N0M0        | II A| well              |                |
| 2           | 64/M       | T1N0M0        | I   | moderately        |                |
| 3           | 65/F       | T3N0M0        | II A| moderately        |                |
| 4           | 67/M       | T2N1M0        | II B| well              |                |
| 5           | 68/M       | T3N1M1b       | IV B| poorly            |                |
| 6           | 70/M       | T3N1M1a       | IV A| well              |                |
| 7           | 70/M       | T3N0M0        | II A| well              |                |
| 8           | 76/M       | T2N1M0        | II B| poorly            |                |
| 9           | 55/M       | T2N1M1a       | IV A| moderately        |                |
| 10          | 55/M       | T1N1M1b       | IV B| poorly            |                |
| 11          | 59/M       | T2N1M0        | II B| poorly            |                |
| 12          | 68/M       | T1N0M0        | I   | moderately        |                |
| 13          | 75/M       | T1N1M0        | II B| poorly            |                |
| 14          | 77/F       | T3N1M1a       | IV A| moderately        |                |

Expression of MK protein was analyzed with anti-MK antibody. TNM was based on the classification by the International Union Against Cancer.

**RESULTS**

**Immunohistochemical analysis** Human specimens were surgically obtained from 14 Japanese esophageal cancer patients (summarized in Table I). The samples were fixed with 4% paraformaldehyde/phosphate-buffered saline at 4°C. The sections of 4–5-µm thickness were incubated with 10% skim milk for 20 min, and then with affinity-purified rabbit anti-MK antibody for 60 min. The sections were sequentially reacted with 0.3% H2O2/methanol, biotinylated anti-rabbit IgG, avidin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) and 3,3-diaminobenzidine; they were washed with phosphate-buffered saline after each reaction. The sections were counterstained with hematoxylin.

**Northern blot analysis** Poly A+ mRNA was extracted using an mRNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). One microgram of mRNA was subjected to electrophoresis in a denaturing formaldehyde-agarose gel and transferred to a nylon filter. The human MK or human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (see Fig. 4) and used in a solution of 50% formamide/5× SSC/50 mM NaH2PO4/2× Denharts/solution/0.1% sodium dodecyl sulfate (SDS)/0.1 mg/ml salmon sperm DNA at 42°C for 12 h. The filter was washed with a solution of 0.2× SSPE/0.1% SDS several times at 50°C.

**Chloramphenicol acetyltransferase (CAT) assay** Human genomic DNA containing the first exon of MK and the 5'-upstream flanking region (2.3 kb) was inserted into pCAT-basic vector (Promega, Madison, WI). Two plasmids, CAT gene driven by the SV40 early promoter and CAT gene without promoters, were from Promega. Esophageal cancer cells were transfected with the respective plasmid DNAs containing the CAT gene (10 µg) and 1 µg of DNA which could constitutively express the β-galactosidase gene (pCH110, Amersham Pharmacia Biotech) using Lipofectin reagent (Life Technologies, Gaithersburg, MD). Two days later, cell extracts were prepared by sonication. To normalize the transfection efficiency, the amounts of cell extracts used for CAT assay were adjusted according to the β-galactosidase activity. CAT activity was measured by the incubation of each extract with 50 µCl of [14C]chloramphenicol and 1 µM acetyl CoA in 0.25 M Tris·Cl (pH 7.8) for 120 min at 37°C as described. Assay of in vitro sensitivity to ganciclovir (GCV) HSV-TK cDNA (from Dr. K. Ikenaka, National Institute for Physiological Science, Okazaki) was ligated into a retrovirus vector LXSN (from Dr. A. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) next to a 2.3 kb genomic DNA fragment (see Fig. 4) and used in CAT assay. Esophageal and melanoma cells were transfected with the vector DNA bearing the MK+HSV-TK gene using Lipofectin reagent and were cultured in G418 (Life Technologies)-containing medium to obtain stably transfected cells. The G418-resistant or wild-type cells, placed in 96-well plates at the density of 5×10^3 cells/well, were cultured in the absence or presence of various concentrations of GCV (0.1–100 µM) for 7 days. The viable cell number in each well was measured with a cell counting kit (Dojindo Laboratories, Kumamoto). The amounts of formazan produced were determined from the absorbance at 450 nm.

**Expression of the MK gene in human esophageal cell lines** In order to establish an experimental system using cell lines, we examined the expression of the MK gene in 7 human esophageal cancer cell lines by northern blot analysis. All the lines tested expressed the transcripts but the amounts of the transcript varied among the lines (Fig. 1).
2). In contrast, a human melanoma line, A875, was negative for midkine expression, and a human fibroblast line, HFF, expressed the MK gene but the level of the expression was markedly lower than that of esophageal lines tested (Fig. 2).

**Promoter activity in human esophageal tumor cells** To investigate whether the 5′-upstream region of MK genomic DNA fragment can be used as a transcriptional promoter in human esophageal tumors, we tested CAT activity in 7 esophageal cancer cell lines transfected with the CAT gene-containing plasmid. All the lines tested could transcribe the reporter gene under the control of 2.3 kb MK genomic region (Fig. 3). However, the transcriptional activities varied among the lines and were not related with the amounts of the MK transcript (Fig. 2). Relative CAT activity powered by the putative MK promoter was less than that of the SV40 promoter in 5 lines. The activity of the MK promoter judged in terms of acetylation rate was 6.0–30% of that of the SV40 promoter. In contrast, 2 lines (TE13 and T.Tn) showed CAT activity comparable to that of the SV40 promoter (85–160%).

**In vitro sensitivity** We then examined the antitumor effect in terms of the expression of the HSV-TK gene controlled by the putative MK promoter. To avoid the effect of the promoter present in the 5′-long terminal repeat of the retrovirus vector, the putative MK promoter-driven HSK-TK gene was inserted in an antisense orientation opposite to that of the retroviral promoter (Fig. 4A).20) The plasmid was then transfected into TE13, TE1 and A875 cells, and the pooled G418-resistant cells were tested for their sensitivity to GCV. The transfected TE13 and TE1 cells showed increased sensitivity compared with the respective parent cells (Fig. 4, B and C), whereas the sensitivity of MK-negative A875 cells remained the same as that of untransfected cells (Fig. 4D). In each experiment, the cells transfected with control DNA bearing the HSV-TK gene without the putative MK promoter showed
the same sensitivity as found in wild-type cells (data not shown).

**DISCUSSION**

In this study we have shown that human esophageal tumors express MK. At the transcriptional level all the human esophageal cancer cell lines tested expressed a large amount of MK mRNA compared with a melanoma and a fibroblast line (Fig. 2). In immunohistochemical staining, more than half of the human specimens from esophageal tumors reacted with anti-MK antibody, in contrast to the surrounding normal esophageal tissues (Fig. 1). Aridome et al. analyzed the MK expression in 2 human esophageal tumors and detected the transcript in tumors, but not in non-cancerous regions.\(^2\) We did not find any correlation between the MK expression in esophageal tumors and the clinical outcome of the patients (data not shown). However, high MK expression in tumors was shown to be a poor prognostic marker in neuroblastoma\(^6\) and bladder cancer patients.\(^8\) Further investigations are necessary to assess the clinical value of the MK expression in esophageal cancer.

Recently we have shown that forced expression of the MK gene could transform NIH3T3 cells\(^2\) and that MK expression was elevated in the early stage of carcinogenesis in human colorectal cancer.\(^2\) However, the biological significance of the MK expression in tumor cells remains unclear. Nevertheless, the 5′-flanking region of the MK gene could be useful for tumor-specific expression. Usefulness of the expression of the HSV-TK gene in tumors, followed by GCV administration, has been investigated in various animal models\(^\) and its clinical application is being tested.\(^\) We found that the 2.3 kb-upstream region of the MK gene contained a promoter activity which could initiate transcription of the fused CAT gene in esophageal cancer cell lines (Fig. 3). Among the lines tested, two (TE13, T.Tn) showed strong transcriptional activity which was comparable to that of the SV40 promoter. A cytotoxic effect caused by GCV was seen in 2 cell lines, TE13 and TE1, when they were transfected with the HSV-TK gene driven by the MK promoter (Fig. 4, B and C). However, their CAT activity generated by the putative MK promoter was not correlated with their sensitivity (Fig. 3). A low level of promoter activity may be enough to transcribe the HSV-TK gene and to convert GCV to phosphorylated GCV. The specificity of MK-based transcription was confirmed by the finding that MK-negative A875 cells did not show increased sensitivity to GCV after transfection with MK-driven HSV-TK gene (Fig. 4D). We observed discordant results between the amount of transcript and the promoter activity in esophageal cancer cell
lines (Figs. 2 and 3). Since the CAT assay solely detects cis-acting elements, the steady-state level of the transcript may be influenced by other elements.

Analysis of the promoter region of the MK gene revealed that it contained a retinoic acid-responsive element. Upregulation of the transcriptional activity by retinoic acid can be observed in tumor cells. The expression of the WTI gene, a tumor suppressor gene identified in Wilms’ tumor, was shown to suppress the MK expression and two possible WTI binding sites were identified in the promoter region. This is an intriguing clue to the mechanism of elevated expression of the MK gene in Wilms’ tumor. However, the expression of the WTI gene is restricted and expression in the esophagus has not been reported. Recently, loss of WT1 expression in colon carcinoma and in breast cancer, whose MK expression was elevated, were demonstrated. Therefore, the biological relevance of the expression of WT1 and that of MK requires further study.

Esophageal tumors occur frequently in aged patients, in whom intensive therapeutic maneuvers may be inappropriate. Several strategies such as combinations of conven-
tional therapies have had limited success in improving the prognosis and the quality of life of patients. The present study suggests a possible gene therapy using a suicide gene/prodrug system. Tumor-specific expression of suicide gene(s) followed by the administration of prodrug(s) can destroy tumor cells without causing serious damage to normal tissues. Obstruction of the esophagus, a complication often found in patients, might be treatable in part by endoscopic injection of vectors bearing the MK promoter-driven suicide gene(s) and non-toxic prodrug administration. The treatment is less invasive than surgery, and should alleviate the patient’s discomfort. Gene therapy with tumor specificity achieved by the usage of the MK promoter, whose activity may be enhanced by retinoic acid, seems to be worth testing in patients with esophageal cancer.

ACKNOWLEDGMENTS

We thank Dr. A. Nakagawara for valuable suggestions and Drs. Y. Koide and H. Matsubara for supplying human specimens. This work was supported by a Grant-in-Aid for Scientific Research and a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan.

(Received September 28, 1998/Revised December 28, 1998/Accepted January 13, 1999)

REFERENCES

1) Kadomatsu, K., Tomomura, M. and Muramatsu, T. 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. Biochim. Biophys. Res. Commun., 151, 1312–1318 (1988).

2) Kadomatsu, K., Huang, R., Suganuma, T., Murata, F. and Muramatsu, T. 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 (1990).

3) Sekiguchi, K., Yokota, C., Asashima, M., Kaname, T., Fan, Q., Muramatsu, T. and Kadomatsu, K. Restricted expression of Xenopus midkine gene during early development. J. Biochem., 118, 94–100 (1995).

4) Michikawa, M., Kikuchi, S., Muramatsu, H., Muramatsu, T. and Kim, S. U. Retinoic acid responsive gene product, midkine (MK), has neurotrophic functions for mouse spinal cord and dorsal root ganglion neurons in culture. J. Neurosci. Res., 35, 530–539 (1993).

5) Muramatsu, H., Shirahama, H., Yonezawa, S., Murata, H. and Muramatsu, T. Midkine, a retinoic acid-inducible growth/differentiation factor: immunochemical evidence for the function and distribution. Dev. Biol., 159, 392–402 (1993).

6) Tsutsui, J., Kadomatsu, K., Matsubara, S., Nakagawara, A., Hamanoue, M., Takao, S., Shimizu, H., Ohi, Y. and Muramatsu, T. A new family of heparin-binding growth/differentiation factors: increased midkine expression in Wilms’ tumor and other human carcinomas. Cancer Res., 53, 1281–1285 (1993).

7) Garver, R. I., Jr., Radford, D. M., Donis-Keller, H., Wick, M. R. and Milner, P. G. Midkine and pleiotrophin expression in normal and malignant breast tissue. Cancer, 74, 1584–1590 (1994).

8) Nakagawara, A., Milbrandt, J., Muramatsu, T., Deuel, T. F., Zhao, H., Cnaan, A. and Brodeur, G. M. Differential expression of pleiotrophin and midkine in advanced neuroblastomas. Cancer Res., 55, 1792–1797 (1995).

9) Choudhuri, R., Zhang, H., Donnini, S., Ziche, M. and Bicknell, R. An angiogenic role for the neurokines midkine and pleiotrophin in tumorigenesis. Cancer Res., 57, 1814–1819 (1997).

10) O’Brien, T., Cranston, D., Fuggle, S., Bicknell, R. and Harris, A. L. The angiogenic factor midkine is expressed in bladder cancer, and overexpression correlates with a poor outcome in patients with invasive cancers. Cancer Res., 56, 2515–2518 (1996).

11) Hurt, R. Surgical treatment of carcinoma of the esophagus. Thorax, 46, 526–535 (1991).

12) Aridome, K., Tsutsui, J., Takao, S., Kadomatsu, K., Ozawa, M., Aikou, T. and Muramatsu, T. Increased midkine gene expression in human gastrointestinal cancers. Jpn. J. Cancer Res., 86, 655–661 (1995).

13) Moolton, F. L. Drug sensitivity (“suicide”) genes for selective cancer chemotherapy. Cancer Gene Ther., 1, 279–287 (1994).

14) Tokunaga, K., Nakamura, Y., Sakata, K., Fujimori, K., Ohkubo, M., Sawada, K. and Sakiyama, S. Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. Cancer Res., 47, 5616–5619 (1987).

15) Uehara, K., Matsubara, S., Kadomatsu, K., Tsutsui, J. and Muramatsu, T. Genomic structure of human midkine (MK), a retinoic acid-responsive growth/differentiation factor. J. Biochem., 111, 563–567 (1992).

16) Herbomel, P., Bourachot, B. and Yaniv, M. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell, 39, 653–662 (1984).

17) Gorman, C. M., Moffat, L. F. and Howard, B. H. Recombinant genomes which express chloramphenicol acetyl-transferase in mammalian cells. Mol. Cell. Biol., 2, 1044–1051 (1982).

18) Miller, A. D. and Rosman, G. J. Improved retroviral vectors for gene transfer and expression. BioTechniques, 7, 980–990 (1989).
19) Vile, R., Miller, N., Chernajovsky, Y. and Hart, I. A comparison of the properties of different retroviral vectors containing the murine tyrosinase promoter to achieve transcriptionally targeted expression of the HSVtk or IL-2 genes. *Gene Ther.*, **1**, 307–316 (1994).

20) Correll, P. H., Colilla, S. and Karlsson, S. Retroviral vector design for long-term expression in murine hematopoietic cells *in vitro*. *Blood*, **84**, 1812–1822 (1994).

21) Kadomatsu, K., Hagihara, M., Akhter, S., Fan, Q.-W., Muramatsu, H. and Muramatsu, T. Midkine induces the transformation of NIH3T3 cells. *Br. J. Cancer*, **75**, 354–359 (1997).

22) Ye, C., Qi, M., Fan, Q.-W., Ito, K., Akiyama, S., Kasai, Y., Matsuyama, M., Muramatsu, T. and Kadomatsu, K. Expression of midkine in the early stage of carcinogenesis in human colorectal cancer. *Br. J. Cancer* (1999), in press.

23) Ram, Z., Culver, K. W., Walbridge, S., Blaese, R. M. and Oldfeld, E. H. *In situ* retroviral-mediated gene transfer for the treatment of brain tumors in rats. *Cancer Res.*, **53**, 83–88 (1993).

24) Vile, R. G., Nelson, J. A., Castleden, S., Chong, H. and Hart, I. R. Systemic gene therapy of murine melanoma using tissue specific expression of the HSVtk gene involves an immune component. *Cancer Res.*, **54**, 6228–6234 (1994).

25) Roth, J. A. and Cristiano, R. J. Gene therapy for cancer: what have we done and where are we going? *J. Natl. Cancer Inst.*, **89**, 21–39 (1997).

26) Pedraza, R. C., Matsubara, S. and Muramatsu, T. A retinoic acid-responsive element in human midkine gene. *J. Biochem.*, **117**, 845–849 (1995).

27) Adachi, Y., Matsubara, S., Pedraza, C., Ozawa, M., Tsutsui, I., Takamatsu, H., Noguchi, H., Akiyama, T. and Muramatsu, T. Midkine as a novel target gene for the Wilms’ tumor suppressor gene (WT1). *Oncogene*, **13**, 2197–2203 (1996).

28) Fraizer, G. C., Wu, Y. J., Hewitt, S. M., Maity, T., Ton, C. C., Huff, V. and Saunders, G. F. Transcriptional regulation of the human Wilms’ tumor gene (WT1). *J. Biol. Chem.*, **269**, 8892–8900 (1994).

29) Hiltunen, M. O., Koistinaho, J., Alhonen, L., Myöhänen, S., Marin, S., Kosma, V.-M., Pääkkönen, M. and Jänne, J. Hypermethylation of the WT1 and calcitonin gene promoter regions at chromosome 11p in human colorectal cancer. *Br. J. Cancer*, **76**, 1124–1130 (1997).

30) Silberstein, G. B., Van Horn, K., Strickland, P., Roberts, C. T., Jr. and Daniel, C. W. Altered expression of the WT1 Wilms tumor suppressor gene in human breast cancer. *Proc. Natl. Acad. Sci. USA*, **94**, 8132–8137 (1997).