Expression of herpes simplex virus-thymidine kinase gene controlled by a promoter region of the midkine gene confers selective cytotoxicity to ganciclovir in human carcinoma cells

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EXPRESSION OF HERPES SIMPLEX VIRUS-THYMIDINE KINASE GENE CONTROLLED BY A PROMOTER REGION OF THE MIDKINE GENE CONFERS SELECTIVE CYTOTOXICITY TO GANCICLOVIR IN HUMAN CARCINOMA CELLS

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A selective expression of suicide gene(s) in tumor cells should produce a preferential cytotoxic effect on tumors. Promoter region(s) of a gene that is expressed in tumors but not in normal tissues can be useful for tumor-specific transcription of a suicide gene. Midkine (MK), a growth/differentiation factor, is expressed predominantly in various types of human tumors, whereas its expression in adult normal tissues is highly restricted. In our study, we showed that a 2.3-kb fragment of genomic DNA in the 5′ upstream region of the MK gene could activate transcription of a fused reporter gene in MK-positive cells but not in MK-negative cells. Efficiency of the *cis*-acting sequence to permit expression of an exogenous gene in tumor cells was comparable with that of the SV40 promoter. Regulated expression of the herpes simplex virus-thymidine kinase (HSV-TK) gene under the control of the MK promoter conferred increased sensitivity to ganciclovir (GCV) on MK-positive tumor cells. Administration of GCV into nude mice that were implanted with MK-positive tumor cells that expressed the HSV-TK gene under the control of the MK promoter could suppress the subsequent tumor growth. Expression of therapeutic genes restricted to tumors can be achieved by the use of the putative *cis*-acting MK promoter.

Key words: *midkine; tumor promoter; gene therapy; HSV-TK*

Tissue-targeted gene expression is an important issue for improvement of safety in gene therapy. Preferential expression of a suicide gene in tumor cells contributes to the safety and the efficacy of suicide gene therapy because nonspecific cytotoxicity to healthy cells can be minimized.[1] *Cis*-acting control regions of the genes, which are transcribed predominantly in tumor cells such as α-fetoprotein,[2,3] carcinoembryonic antigen,[4,5] DF3/MUC1,[6] ErbB-2,[7] and prostate-specific antigen gene,[8] have been tested for their capacity to activate transcription of a suicide gene. Although the use of these promoters can enable tumor cells to express therapeutic gene(s) in a restricted manner, scope of tumors to which these promoters are applicable is relatively limited.

Midkine (MK) is a retinoic acid-responsive growth/regulatory factor that was originally identified from a germ line tumor.[9] Expression of the MK gene in human adult tissues is low and is restricted to lung alveoli, kidney and mucosal tissues of the gastrointestinal tract.[10] However, increased expression was observed in a variety of human tumors such as neuroblastomas,[11] lung carcinomas,[12] bladder carcinomas,[13] breast cancer[14] and gastrointestinal carcinomas.[15,16] Therefore, a *cis*-acting transcriptional control sequence in the MK gene is expected to allow restricted transgene expression in tumor cells.

Among the suicide genes tested, the herpes simplex virus-thymidine kinase (HSV-TK) gene has been extensively investigated for its possible clinical application to cancer patients.[17,18] It can convert an inactive prodrug, ganciclovir (GCV), into a cytotoxic agent, phosphorylated GCV. In our study, we examined whether a 5′ upstream genomic DNA fragment of the MK gene can be used to transcribe the HSV-TK gene effectively and preferentially in MK-positive tumor cells.

MATERIAL AND METHODS

Cells and animals

QG56 (human lung carcinoma), NGP (human neuroblastoma), AsPC-1 (human pancreatic carcinoma), A875 (human melanoma) and HFF (human foreskin fibroblast) cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS. BALB/c *nu/nu* nude mice (6- to 8-week-old females) were purchased from Japan SLC (Hamamatsu, Japan).

Northern blot analysis

Poly $(A)^+$ mRNA (1 μg) was subjected to electrophoresis in a formaldehyde-agarose gel and transferred to a nylon filter. MK, HSV-TK and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA[19] were labeled with [^{32}P]dCTP and used for probes. Hybridization was performed in a solution of 50% formamide/5× SSC/50 mM NaH_2PO_4/2× Denharts' solution/0.1% SDS/0.1 mg/ml salmon sperm DNA at 42°C for 12 hr. The filters were washed with a solution of 0.2× SSPE/0.1% SDS several times at 50°C.

CAT assay

Human MK genomic DNA containing 5′ upstream flaking region (−2285 to +34), which includes the first exon,[20,21] was inserted into a pCAT-basic vector (Promega, Madison, WI) (MKp-CAT). Two plasmids, the CAT gene whose expression was driven by the SV40 immediate early promoter (SV40-CAT) and the CAT gene without promoters (pCAT-basic), were used as controls. Cells were transfected with 10 μg of the respective plasmid DNA and 1 μg of the β-galactosidase gene expression vector DNA

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(pCH110, Amersham Pharmacia, Uppsala, Sweden) using lipofectin reagent (Life Technologies, Gaithersburg, MD). Two days later, cell extracts were prepared by sonication. To standardize transfection efficiency, the amounts of cell extracts for CAT assay was quantitated with the β-galactosidase activity.[22] CAT activity was measured by the incubation of each extract with 50 μCi of [^{14}C] chloramphenicol and 1 mM acetyl CoA in 0.25 M Tris-HCl (pH 7.8) for 30 min at 37°C.[23] Each CAT assay was performed in triplicate.

In vitro GCV sensitivity assay

The HSV-TK gene or fused HSV-TK gene with the 2.3-kb MK genomic DNA was inserted into a retrovirus vector LXSN (provided by Dr. A.D. Miller, Seattle, WA)[24] in the opposite direction to the long terminal repeat (TK-R and MKp-TK, Fig. 3*a*). Cells were transfected with the plasmid DNA, and G418-resistant cells were selected. These cells were seeded in 96-well plates at the density of 5×10^2 cells/well and cultured with various concentrations of GCV. After 5–7 days, viable cell number in each well was measured using a cell-counting kit (Wako, Osaka, Japan). The amount of formazan produced in each well was determined from the absorbance at 450 nm.

Animal study

Parent and transfected QG56 cells (5×10^6) with the MKp-TK DNA or the TK-R DNA were inoculated subcutaneously into nude mice ($n = 7$). Tumor volumes were calculated according to the formula, tumor volume $= 0.5 \times a \times b$,^{2} where a and b are the larger and the smaller diameters, respectively. When tumor vol-

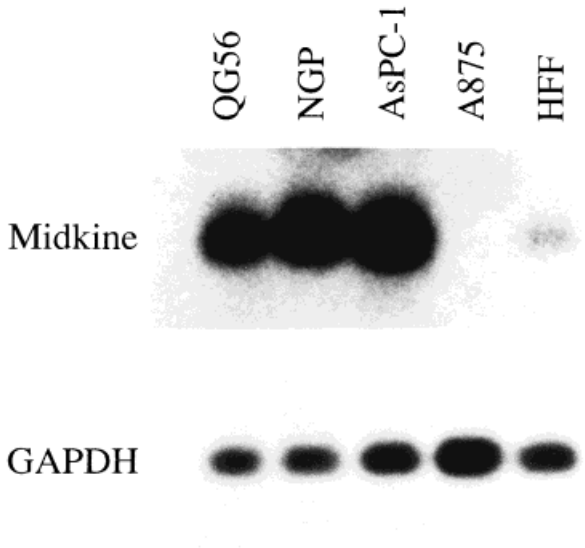

FIGURE 1 – Expression of the MK gene in human cancer cells (QG56, NGP, AsPC-1 and A875) and fibroblasts (HFF). The same Northern blot was rehybridized with a probe of GAPDH as a control.

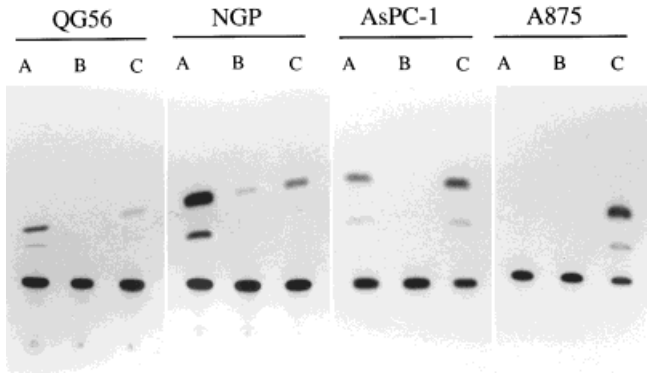

FIGURE 2 – A representative autoradiogram of a CAT assay in human cancer cells. (Lane A) The MK promoter fused with the CAT gene (MKp-CAT), (lane B) pCAT-basic or (lane C) the SV40 promoter fused with the CAT gene (SV40-CAT) was transfected into respective cells.

TABLE I – RELATIVE RATIOS OF THE CAT ACTIVITY DRIVEN BY THE MK PROMOTER AND BY THE SV40 IMMEDIATE EARLY PROMOTER IN TUMOR CELLS

Cells tested	MKp-CAT / SV40-CAT	Average ± SE
QG56	1.2, 4.8, 4.8	3.6 ± 1.2
NGP	3.6, 4.7, 5.5	4.6 ± 0.56
AsPC-1	0.8, 0.9, 0.9	0.87 ± 0.033
A875	0.05, 0.07, 0.07	0.063 ± 0.007

Relative ratios of the CAT activity in transfected cells are shown after standardization of the transfection efficiency.

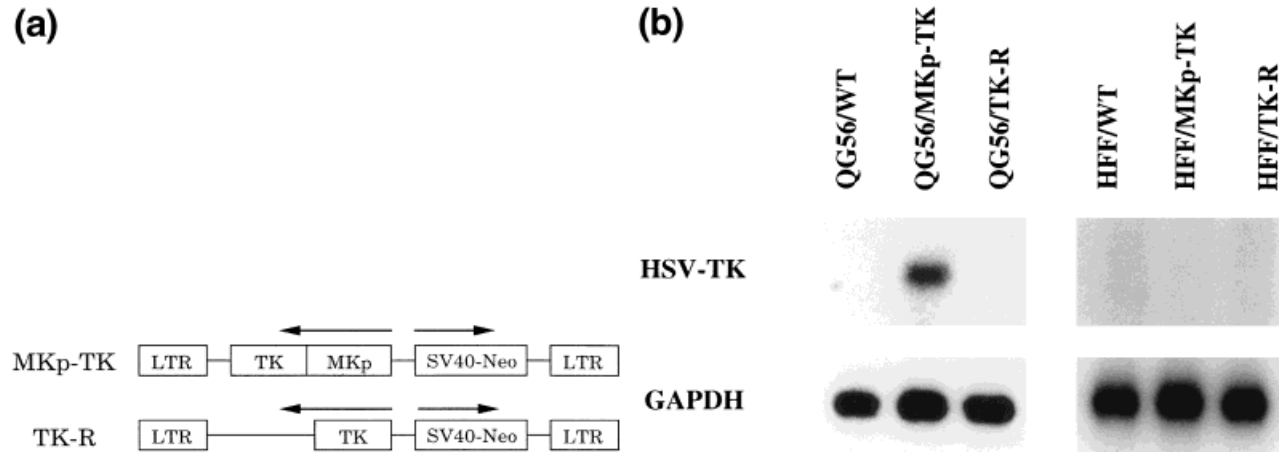

FIGURE 3 – *(a)* Retroviral vectors for transfection. The HSV-TK gene was placed in the opposite direction of the long terminal repeat with (MKp-TK) or without (TK-R) the MK promoter. The arrows indicate the orientation of transcription. *(b)* Northern blot analysis showing the expression of the HSV-TK gene in parent QG56 (QG56/WT) and HFF (HFF/WT) cells, in transfected QG56 and HFF cells with either the MKp-TK (QG56/MKp-TK, HFF/MKp-TK) or the TK-R DNA (QG56/TK-R, HFF/TK-R). The same blot was rehybridized with a probe of GAPDH as a control.

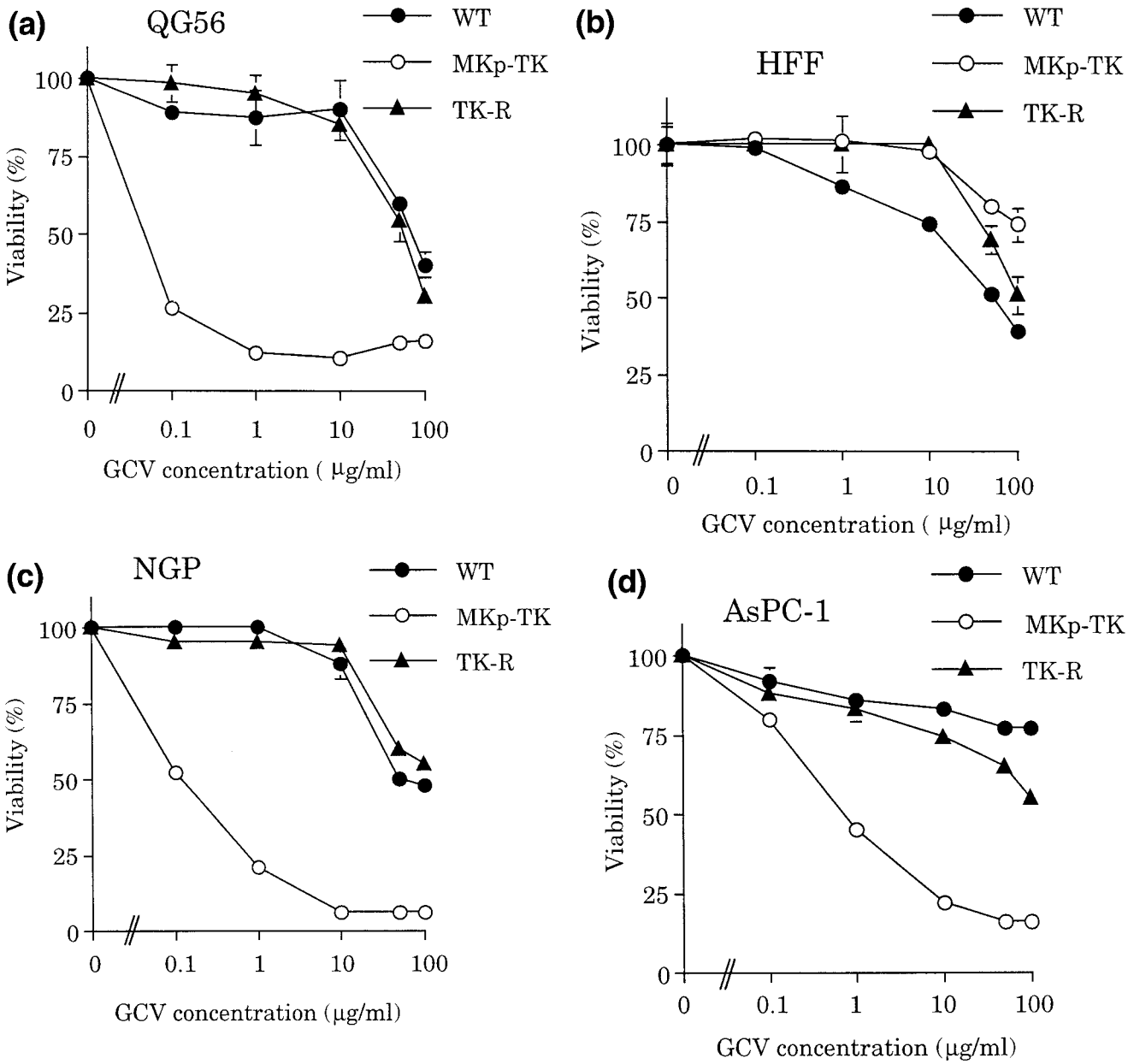

FIGURE 4 – *In vitro* sensitivity of QG56 *(a)*, HFF *(b)*, NGP *(c)* or AsPC-1 *(d)* cells to GCV. Parent (WT) cells (●) or cells transfected with either the MKp-TK (○) or the TK-R (▲) DNA were tested at various concentrations of GCV. Standard error bars are shown.

umes reached 100 mm^3 (day 12 after tumor inoculation), GCV (30 mg/kg/day) or saline as a control was intraperitoneally administered for 5 days.

RESULTS

Expression of the MK gene

We examined the expression of the MK gene in several cell lines (Fig. 1). Three human tumor cell lines, QG56, NGP and AsPC-1, expressed the MK gene, whereas the expression in HFF cells was significantly low. A875 cells were negative for MK expression.

Transcriptional activity mediated by the MK genomic region

We tested whether a 2.3-kb genomic DNA fragment in the 5′ upstream region of the MK gene[20] could activate a fused chloramphenicol acetyltransferase (CAT) reporter gene in MK-positive cells using the MKp-CAT DNA. To estimate relative promoter activity that the 2.3-kb fragment possesses, SV40-CAT DNA was also transfected. The CAT assay showed that the CAT activity mediated by the MK fragment was greater than that of the SV40 promoter in QG56 and NGP cells and was comparable with that of the SV40p-CAT DNA in AsPC-1 cells (Fig. 2 and Table I). In contrast, the transcriptional activation of the CAT gene tested with the MKp-CAT DNA was scarcely detected in MK-negative A875 cells (Fig. 2 and Table I).

Sensitivity of transfected tumor cells with the MKp-TK DNA to GCV

In an attempt to express the HSV-TK gene preferentially in tumors, the suicide gene was designed to be driven by the MK promoter (MKp-TK) using a retroviral vector. To avoid the effect of the viral promoter located in the long terminal repeat, the MKp-TK construct was inserted in the orientation opposite to the

viral promoter (Fig. 3*a*). Cells were transfected with either the MKp-TK or the TK-R (HSV-TK gene without promoters) DNA (Fig. 3*a*), and respective G418-resistant cells were obtained. *In vitro* proliferation rate and morphology were not markedly different between the transfected and parent cells. Expression of the HSV-TK gene was detected in QG56 cells that were transfected with the MKp-TK DNA (QG56/MKp-TK cells) but not in the transfected cells with the TK-R DNA (QG56/TK-R cells) (Fig. 3*b*). In contrast, HSV-TK transcripts were undetectable when HFF cells expressing the MK gene at a low level (Fig. 1) were transfected with the MKp-TK DNA (Fig. 3*b*). Sensitivity to GCV was consequently increased in QG56/MKp-TK cells compared with that in QG56/TK-R or parent cells (Fig. 4*a*). On the other hand, HFF cells did not show increased sensitivity to GCV (Fig. 4*b*). Other MK-positive tumor cells, NGP and AsPC-1 cells, became susceptible to GCV compared with parent cells when they were transfected with the MKp-TK but not with the TK-R DNA (Fig. 4*c,d*).

GCV-mediated regression of tumors that expressed the HSV-TK gene driven by the MK promoter

Parent QG56 and QG56/MKp-TK or QG56/TK-R cells were subcutaneously inoculated at different sites of the same nude mice. Mice developed both types of tumors with a similar growth rate. When the tumors reached 100 mm^3 in volume, mice were intraperitoneally injected with either GCV or saline as a control. Administration of GCV resulted in significant suppression of subsequent growth of QG56/MKp-TK tumors and all of the tumors disappeared (Fig. 5*a*). In contrast, growth of parent or QG56/TK-R tumors was temporally retarded by administered GCV but the tumors regrew thereafter (Fig. 5*a*). Injection of saline did not affect the growth of parent or QG56/MKp-TK tumors (Fig. 5*b*).

DISCUSSION

Up-regulation of the MK expression has been shown in various kinds of human tumors.[11–15] We have also found that most of the human lung cancer cell lines (9 of 10 lines) (data not shown) and all of the esophageal cancer lines tested (7 lines) expressed the MK gene,[16] whereas the expression levels in several human fibroblast cell lines were markedly low (data not shown). Mechanism of this up-regulation in a number of tumors remains unclear, but the amount of MK transcript was not correlated with the rate of cell proliferation (manuscript in preparation). Although functional roles of MK in tumorigenesis are not well understood, forced expression of the MK gene induced transformation of NIH3T3 cells[25] and enhanced angiogenesis through increased proliferation rate of endothelial cells.[26] Recently, we found that MK expression level was elevated even at the early stage of carcinogenesis of human colorectal cancer.[27]

Specificity of the MK promoter-mediated transcription was shown in our study. The MK promoter drove the fused CAT reporter gene in MK-positive tumor cells, and the sensitivity of their MKp-TK-transfected cells to GCV was increased (Figs. 2 and 4*a,c,d*). In contrast, the MK promoter did not activate the fused CAT gene in MK-negative A875 cells (Fig. 2 and Table I), and the low MK expression level of HFF cells (Fig. 1) correlated with undetectable expression level of the HSV-TK gene in the HFF/MKp-TK cells (Fig. 3*b*) and their reduced sensitivity to GCV (Fig. 4*b*). The amounts of the MK transcript didn't correspond as well to the transcriptional activity of the MK promoter, as shown in the case of AsPC-1 cells (Fig. 1 and Table I). However, the MK promoter activity tested in CAT assay correlated with the level of increased sensitivity to GCV because the susceptibility of AsPC-1/MKp-TK cells were less significant compared with QG56/MKp-TK or NGP/MKp-TK cells (Fig. 4). Sequence analysis of the promoter region revealed the presence of several potential binding sites for transcriptional factors such as retinoic acid receptors[21] and the product of Wilms' tumor-suppressor gene,[28] but these might not explain the preferential expression of the MK gene in tumor cells. Moreover, retinoic acid did not modulate the MK promoter-driven transcriptional activity (data not shown). To narrow down a region that is responsible for the expression restricted to tumor cells, reporter gene assays using sequentially deleted fragments from the 5′ upstream region are currently being performed. The minimal DNA fragment, when identified, should contribute to construction of a vector that can improve the specificity and the efficacy of transcriptional control in tumors.

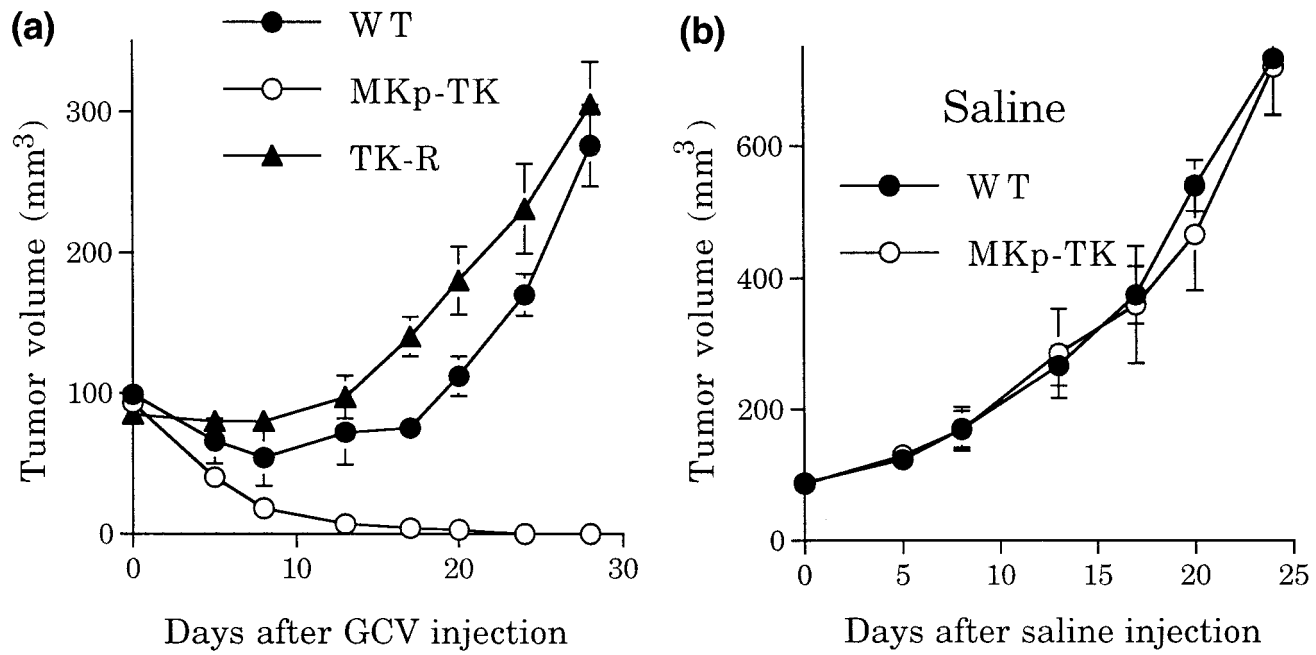

FIGURE 5 – Injection of GCV *(a)* or saline *(b)* into mice that developed tumors of parent QG56 cells (WT) (●) or transfected QG56 cells with the MKp-TK (○) or the TK-R (▲) DNA. Standard error bars are shown.

Besides the preferential expression in tumor cells, the transcriptional activity that the MK promoter possesses in MK-positive cells is as strong as that of the SV40 promoter (Table I). Moreover, CAT activity controlled by the MK genomic region was stronger than that of the c-erb B2 promoter region[29] in breast cancer cells (data not shown). Our preliminary study, however, showed that CAT activity regulated by the MK promoter is weaker than that of the cytomegalovirus promoter. Nevertheless, the MK promoter is potent enough to drive the HSV-TK gene in MK-positive cancer cells and could be a useful tool for suicide gene therapy. For clinical application of this promoter system, adenovirus bearing the MKp-TK construct is to be tested because it enables us to examine a differential damaging effect on tumors and surrounding normal tissues *in vivo*. Since secreted MK, as a tumor-associated marker, can be detected in cancer patients with enzyme-linked immunoassay,[30] candidate patients for MK-based suicide gene therapy can be appropriately selected.

In summary, we have shown that the promoter region of the MK gene contained a *cis*-acting sequence that could limit the expression of transgene to tumor cells. A suicide gene therapy with a broad therapeutic window can be potentially applicable to a variety of cancer patients based on the transcriptionally targeted vector system presented in our study.

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