Jpn. J. Cancer Res. 88, 678–686, July 1997

Usefulness of a Mouse Myelin Basic Protein Promoter for Gene Therapy of Malignant Glioma: Myelin Basic Protein Promoter Is Strongly Active in Human Malignant Glioma Cells

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We have searched for suitable promoters to regulate the expression of suicide genes for use in gene therapy. We have shown that the 1.3-kb fragment of the mouse myelin basic protein (MBP) promoter region initiates transcription in mouse glioma cells more efficiently than glial fibrillary acidic protein (GFAP) or myelin proteolipid protein (PLP) promoter. Among three different lengths of the MBP promoter, the shortest (256-bp) core promoter region initiates transcription as efficiently as 650-bp or 1.3-kb MBP promoter lengths in RSV-M glioma cells. To assess the suitability of the MBP promoter for use in clinical trials of malignant glioma gene therapy, we also had to show that it (the 1.3-kb length in this case) is effective in human glioma cells, as well as in murine glioma cells. The activity of the MBP promoter is much higher than that of GFAP or PLP promoter in most human glioma cells, suggesting that the MBP promoter would be best for directing toxic gene expression in gene therapy for patients with malignant glioma. Human glioma cells in which the MBP promoter was strongly active were sensitive to ganciclovir when they were transduced with MBP promoter/herpes simplex virus thymidine kinase gene-bearing retroviruses. In conclusion, retrovirus-targeted gene therapy for malignant glioma using this MBP promoter is a promising candidate for clinical trials.

Key words: Malignant glioma — Gene therapy — MBP promoter — Ganciclovir

Retrovirally mediated gene therapy for malignant glioma has been one of the most reliable genetic manipulations among those tried for various diseases ranging from cancer to AIDS, because retroviruses integrate into actively dividing cells but not into nondividing cells.[1] It is therefore possible to kill selectively glioma cells without causing toxicity to normal neurons or astrocytes by selectively transferring toxic genes into glioma cells.[2,3] Clinical protocols using gene therapy for glioma have already been initiated because of the poor prognosis of this disease despite aggressive therapy.[4] However, if the toxic gene is also expressed in nontumorous dividing cells in the central nervous system (CNS) (e.g., reactive astrocytes or endothelial cells), side effects such as epilepsy or cerebral infarction due to obstruction of vessels may occur. We have therefore searched for suitable promoters to regulate the expression of suicide genes, such as the herpes simplex virus type 1 thymidine kinase (HTK) gene, in order to increase the effectiveness of glioma gene therapy and to decrease its side effects.

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein that is abundant in astrocytes. Antibodies to GFAP selectively stain astrocytes, and are used to identify them. GFAP has also been detected in the glioma cells making up many astrocytomas. In contrast, myelin basic protein (MBP) and myelin proteolipid protein (PLP) are major proteins of myelin. The MBP gene is expressed both in oligodendrocytes in the CNS and Schwann cells in the periphery, while the PLP gene is predominantly expressed in oligodendrocytes. MBP accounts for about 30% of the protein in CNS myelin and 5 to 15% of the protein in peripheral myelin.[5,6] These proteins are oligodendrocyte-specific in the CNS, and there have been few reports of their presence in glioma cells. Therefore, the promoter of the GFAP gene has been considered the best candidate for regulating the suicide gene in retrovirally mediated gene therapy for malignant glioma. Unexpectedly, however, the MBP gene promoter was found to be the best glioma-specific promoter among these three promoters in mouse glioma cells.[7] For instance, the promoter activity of the 1.3-kb flanking sequence of the MBP gene is much higher than that of the GFAP or PLP promoter and the activity is as high as that of the Moloney murine leukemia virus (MoMLV) LTR in RSV-M mouse glioma cells. Gene therapy for glioma using the MBP promoter in recombinant retroviruses thus has the potential to be very effective while having low toxicity to nonneoplastic cells. Since there was a discrepancy between expression of the endogenous MBP promoter and MBP promoter activity carried on the retroviral vector, we dissected the MBP

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promoter to identify *cis*-lying elements that are responsible for efficient transcription initiation in RSV-M glioma cells. Even the shortest 256-bp promoter region of mouse MBP gene tested sufficed to drive efficient transcription in glioma cells.

To apply this MBP promoter/HTK retrovirus to human glioma gene therapy, it is necessary to show that the MBP promoter is also active in most human malignant glioma cells. In this study we assayed the activities of several glial-specific promoters (GFAP, MBP, PLP) in a variety of human glioma cells.

MATERIALS AND METHODS

Plasmid construction All plasmids were constructed by standard recombinant DNA techniques.[8] Construction of pIP200 and pNT230 has been described previously,[9, 10] and Fig. 1 diagrams the structures of GFA2.5/pIP200, MBP1.3/pIP200, MBP650/pIP200, MBP256/pIP200, and PLP1.5/pIP200. These plasmids contain the neomycin phosphotransferase (*neo*R) gene, which confers G418 resistance on transfected cells, and the *Escherichia coli* (*E. coli*) *lacZ* gene as a reporter gene within two MoMLV LTRs. The GFAP (2.5-kb *Hin*d III fragment from pGF1L,[11]) MBP,[12] and PLP promoters were subcloned into the *Hin*d III site and *Sal* I site of pIP200, respectively.[7] To construct MBP650/pIP200, the 1.3-kb *Hin*d III fragment of MBP promoter was inserted into pUC19, and the small *Pst* I fragment was removed and self-ligated (MBP1.3-pUC19Δ). The *Sal* I-*Hin*d III fragment was purified from MBP1.3-pUC19Δ, and this fragment was inserted into the *Sal* I-*Hin*d III site of pIP200 (MBP650/pIP200). To construct MBP256/pIP200, a *Hin*d III linker was inserted into the *Stu* I site of 256 bp of 5′-flanking sequence, and this *Hin*d III fragment was inserted into the *Hin*d III site of pIP200.

Cell lines Human and rat glioma cell lines (Table I) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). NIH-3T3 mouse fibroblasts also were grown in DMEM containing 10% FBS. ONS-12 and ONS-75 are human glioma cell lines that were established from glioblastoma specimens obtained surgically in our department. ONS-76 is a medulloblastoma cell line that also was established in our department.[13] U-87MG, U-118MG, and U-138MG[14] are glioblastoma cell lines obtained from American Type Culture Collection (ATCC). KNS-60, KNS-81 and KNS-89 are established cell lines.[15] The RSV-M mouse glioma cell line has been previously described.[7] Psi-2 cells were kindly provided by Dr. Mulligan and used as ecotropic retrovirus-producing cells.[16] PA-317 fibroblasts were obtained from ATCC and used as amphotropic retrovirus-producing cells.[17]

Assay of β-galactosidase activity Following three washes in phosphate-buffered saline (PBS), transduced cells were harvested using a rubber policeman and resuspended in 100 μl of 0.25 *M* Tris-Cl (pH 7.8). Cells were disrupted by three cycles of freezing in liquid nitrogen and thawing in water at 37°C. The suspension was centrifuged at 12000 rpm for 5 min at 4°C in a microcentrifuge and transferred to a fresh microcentrifuge tube. Fifty microliters of this solution was used for the β-gal assay and another 50 μl for the CAT assay described later. Cell extracts were incubated with 300 μl of 1 m*M* $MgCl_2$, 45 m*M* β-mercaptoethanol, 250 μg of *o*-nitrophenyl-β-D-galactopyranoside, and 67 m*M* sodium phosphate (pH 7.5) at 37°C. After a faint yellow color had developed, 500 μl of 1 *M* Na_2CO_3 was added to stop the reaction, and the $absorbance_{420}$ (A_{420}) was read. When the cells were cotransfected with pSV2CAT, the A_{420} obtained was normalized according to the CAT activity in the same cell extract to exclude differences between transfections.

β-Galactosidase activity in individual cells was measured by the fluorescein-di-β-D-galactopyranoside(FDG)-FACS method described by Nolan *et al.*[18] FDG was

Table I. Summary of the Glioma Cell Lines

Name	Species	Patient's age	Histology	Other features
RSV-M	mouse		anaplastic astrocytoma	induced by Rous sarcoma virus
C6	rat		glial tumor	induced by *N*-nitrosomethylurea; GFAP (+)
U-87MG	human	44	glioblastoma	
U-118MG	human	50	glioblastoma	
U-138MG	human	47	glioblastoma	
KNS-60	human	55	glioma	GFAP (−)
KNS-81	human	65	glioma	GFAP (+)
KNS-89	human	66	gliosarcoma	GFAP (+)
ONS-12	human	67	glioblastoma	GFAP (+)
ONS-75	human	39	anaplastic astrocytoma	
ONS-76	human	2	medulloblastoma	

synthesized according to Rotman *et al.*[19] and the fluorescence intensity was measured using FACS Vantage (Becton Dickinson, Mountain View, CA).

Lipofection of plasmid DNA Glioma cells ($4-8\times 10^4$/cm^2) were incubated in a 100×100 mm dish at 37°C in a CO_2 incubator for 18 to 24 h until they were subconfluent. For each transfection, 10 μg of DNA (BAG, GFA2.5/pIP200, MBP1.3/pIP200 or PLP1.5/pIP200) and 2 μg of pSV2CAT were diluted in 500 μl of DMEM without serum or antibiotics. The MoMLV LTR controls the *lacZ* gene expression and the SV40 early promoter controls the *neo*R gene in the BAG retroviral vector.[20] Ten microliters of LipofectAMINE (Gibco BRL, Grand Island, NY) was diluted into 500 μl of serum-free DMEM, and the solution was added to the DNA-containing solution. This solution was mixed gently and incubated at room temperature for 45 min to allow DNA-liposome complexes to form. Four milliliters of DMEM was added to this solution, and rinsed cells were overlaid with the diluted complex solution. They were incubated for 5 h at 37°C in a CO_2 incubator, then 5 ml of DMEM with 20% FBS was added without removing the transfection mixture. The medium was replaced at 24 h after the start of transfection, and incubation was continued for another 24 h. Cell extracts were harvested 48 to 72 h after the start of transfection, and assayed for gene activity (β-galactosidase and CAT assays).

X-gal staining of β-galactosidase-producing gene-transduced cells Cells were transduced by lipofection as described above, on a smaller scale, cultured for 48 h at 37°C, and fixed with 0.5% glutaraldehyde for 5 min at room temperature. After three washes with PBS containing 1 m*M* $MgCl_2$, cells were incubated in an X-gal reaction mixture containing 0.1% X-gal, 0.01% sodium deoxycholate, 0.02% NP-40, 1 m*M* $MgCl_2$, 5 m*M* $K_3[Fe(CN)_6]$, and 5 m*M* $K_4[Fe(CN)_6]$ in calcium- and magnesium-free PBS.

CAT assay The 50 μl of lysate that was not used for the β-gal assay was heated at 60°C for 15 min to inactivate deacetylase,[21] and centrifuged at 15000 rpm for 5 min. The supernatant was used for determination of CAT activity[22] with some modifications. The normalized β-gal activity was calculated using the formula: normalized β-gal activity (mU/mg/100%CAT) = (β-gal activity/percentage of CAT conversion) $\times$ 100.

***In vitro* sensitivity of retrovirus-transduced glioma cells to ganciclovir** Cultured cells were transduced with MBP1.3/pNT230 (supernatant of PA-317 producing MBP1.3/pNT230 retroviruses), and retrovirally transduced cells were selected by the addition of an appropriate concentration of G418 (ranging from 0.2 mg/ml to 1.2 mg/ml) for about a week. Then these cells were subjected to an *in vitro* assay of sensitivity to ganciclovir (GCV) (F. Hoffmann-La Roche Ltd., Basel; Tanabe Seiyaku, Osaka) as described previously.[7] The viability rate then was calculated using the formula: percent cell survival (%) = (mean experimental A_{540}/mean control A_{540}) $\times$ 100, where control A_{540} is the absorbance of the well in the absence of GCV.

***In vivo* sensitivity of glioma cells transduced with HTK-bearing retroviruses to GCV in nude mice** U-87MG human glioblastoma cells (1×10^7 cells/200 μl PBS) were inoculated subcutaneously into 7-week-old female BALB/c *nu/nu* mice obtained from SLC (Shizuoka). U-87MG cells that had been transduced with MBP1.3/pNT230 and selected by G418 were also inoculated subcutaneously in nude mice. Three days after tumor inoculation, 50 mg/kg GCV or PBS for control mice was injected intraperitoneally once a day for 14 days, and the tumor volumes of both groups were observed. Tumor volume was calculated as follows: tumor volume = (longer diameter) $\times$ (shorter diameter)^2.

RESULTS

A 256-bp promoter region of the mouse MBP gene is sufficient to drive efficient transcription initiation in RSV-M glioma cells We have shown that a 1.3-kb fragment of the mouse MBP promoter region efficiently initiates transcription in mouse glioma cell (RSV-M). However, we could not detect MBP-mRNA in RSV-M glioma cells by northern blotting (data not shown). To identify *cis*-lying elements responsible for this efficient transcription initiation, we made two deletion constructs of the 1.3-kb MBP promoter region (Fig. 1A). A 256-bp promoter region is the shortest MBP-promoter region required for tissue-specific and efficient transcription initiation in several cell lines[23] and in transgenic mice.[11, 24] This 256-bp fragment and a 650-bp fragment of the MBP promoter region were inserted into pIP200 vector, and the plasmids were transfected into Psi-2 cells to obtain recombinant retrovirus. The pIP200 retrovirus carrying the 1.3-kb, 650-bp, or 256-bp MBP promoter region (2.5×10^3 CFU) was transduced into RSV-M glioma cells, and stable transformants were selected by the addition of G418. Beta-galactosidase (β-gal) activities in lysates of transformed RSV-M glioma cells, measured using *o*-nitrophenylgalactoside as a substrate, did not differ significantly from each other (Fig. 2). Since this assay system measures an averaged activity of the stable transformants and may obscure differences in the distribution of β-gal activities among the cells carrying different length promoters, we also measured β-gal activity in each cell using the FDG-FACS method. The distribution of the β-gal activities remained quite similar (Fig. 3). Therefore, the shortest core MBP promoter region was sufficient to initiate efficient transcription in RSV-M glioma cells.

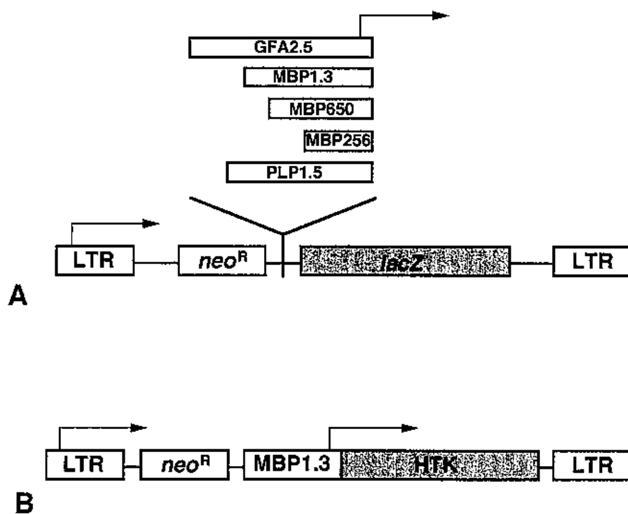

Fig. 1. Construction of the pIP200 series and MBP1.3/pNT230. pIP200 contains the neomycin phosphotransferase (*neo*R) gene and the *E. coli lacZ* gene within two Moloney murine leukemia virus long terminal repeats (LTRs). pNT230 contains the HTK gene instead of the *lacZ* gene of pIP200. Promoters of GFAP (GFA2.5), MBP (MBP1.3, MBP650, MBP256), or PLP (PLP1.5) were inserted into the *Hin*d III or *Sal* I site between the *neo*R and *lacZ* genes (A). The *lacZ* gene was then converted to the 2.8 kb HTK gene, whose expression was directed by the MBP promoter (MBP1.3/pNT230) (B). MBP, myelin basic protein; GFAP, glial fibrillary acidic protein; HTK, herpes simplex type 1 thymidine kinase; PLP, proteolipid protein.

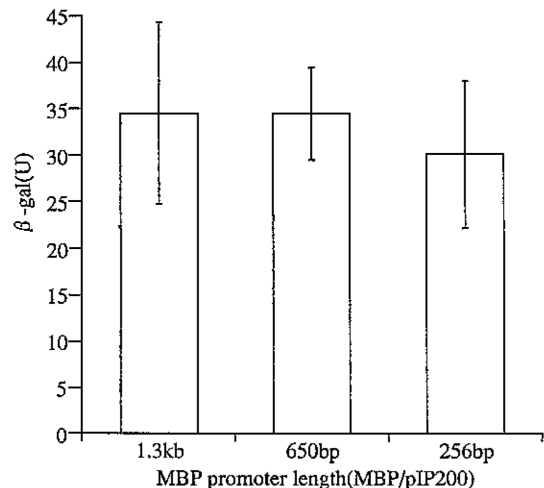

Fig. 2. Promoter activities of the 5′-flanking region of the MBP gene in RSV-M glioma cells. β-Galactosidase activities were measured in lysates of RSV-M glioma cells which were transduced with the *lacZ* gene controlled by 1.3-kb, 650-bp, or 256-bp MBP promoter regions. Bars indicate the standard error of the mean.

Exogenous MBP promoter is also active in many human glioma cells To evaluate the feasibility of general application of the MBP promoter to glioma gene therapy, we needed to know whether the MBP promoter region used in mouse glioma cells can initiate efficient transcription in human malignant glioma cells as well.

We performed β-gal assays on extracts from glioma cells (Table I) that had been transduced by lipofection with plasmid DNA containing the *lacZ* gene controlled by glial-specific promoters, including the 1.3-kb 5′-flanking region of the MBP gene (MBP1.3/pIP200), the 2.5-kb 5′-flanking region of the GFAP gene (GFA2.5/pIP200), and the 1.5 kb 5′- flanking sequence of the PLP gene (PLP1.5/pIP200) (Fig. 1A). We evaluated the β-gal activity relative to CAT activity derived from a cotransfected pSV2CAT plasmid (see "Materials and Methods"). Since our goal is retrovirally mediated gene therapy, promoter activity should be estimated in human glioma cells transduced with retroviruses. Initially, human glioma cells were infected with amphotropic retroviruses that bore the *lacZ* gene produced by PA317 cells. However, X-gal stained less than a few percent of cells despite

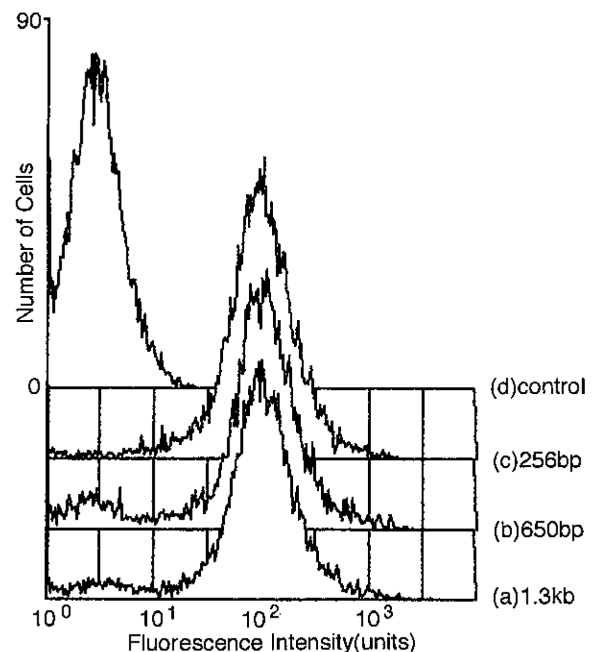

Fig. 3. Distribution of β-galactosidase activities among transformed RSV-M glioma cells carrying three MBP promoter segments of different length. β-Galactosidase activities were measured in RSV-M glioma cells which were transduced with the *lacZ* gene controlled by 1.3-kb (a), 650-bp (b), and 256-bp (c) MBP promoter using the fluorescein-β-D-galactopyranoside (FDG)-FACS method. The distribution of β-galactosidase activity from wild-type RSV-M glioma cells served as a negative control (d).

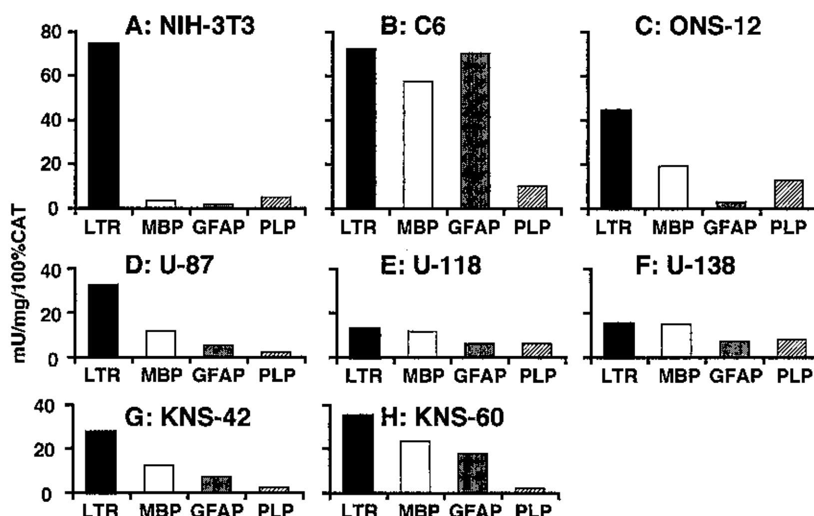

Fig. 4. Promoter activities of LTR, GFAP, MBP and PLP constructs in glioma cells. β-Galactosidase activities normalized on the basis of CAT assays (see "Materials and Methods") were obtained from cell extracts of NIH-3T3 fibroblasts (A), C6 rat glioma cells (B), ONS-12 (C), U-87MG (D), U-118MG (E), U-138MG (F), KNS-42 (G) or KNS-60 human glioma cells (H) which were lipofected with the BAG, GFA2.5/pIP200, MBP1.3/pIP200 or PLP1.5/pIP200 plasmids. Solid bars (the first bars in each graph) reflect LTR; open (second) bars, MBP; gray (third) bars, GFAP; and oblique-lined (fourth) bars, PLP promoter activities. Normalized β-gal activity was measured when transfection efficiency was judged to be 100% per mg for each cell extract (mU/mg/100%CAT).

the BAG retrovirus titer being at 5×10^5 CFU/ml (data not shown). Some of the human glioma cells were not transduced efficiently by the amphotropic retrovirus (less than a few percent of cells transduced), so it was not appropriate to measure the promoter activity from these extracts. In contrast, when the BAG plasmid was transfected with the LipofectAMINE reagent, the prevalence of the blue stain ranged from 20 to 40% of the glioma cells. For the purpose of evaluating promoter activity in human glioma cells, it is not necessary to transduce the *lacZ* gene construct via retroviruses. Therefore, in this study, we chose lipofection instead of retrovirus infection to introduce the genes into glioma cells. A recombinant BAG retrovirus[20] served as a control, in which expression of the *lacZ* gene was driven by the MoMLV 5′-LTR, resulting in β-gal production without any tissue specificity. The LTR promoter was highly active in both NIH-3T3 mouse fibroblasts and C6 rat glioma cells (Fig. 4, A and B). On the other hand, glial-specific promoters were active in rat glioma cells, but not in fibroblasts. In human glioma cells, the MBP promoter was more active than the GFAP or PLP promoter (Fig. 4, C–G). The activity of the MBP promoter was as high as that of LTR promoter in some cell lines (Fig. 4, E and F).

Selective killing by GCV of several human glioma cell lines transduced with retrovirus bearing an MBP promoter-HTK gene Next, we studied the correlation between the sensitivity to GCV and the MBP promoter activity in human glioma cells. Infectious retrovirus particles (MBP1.3/pNT230) were produced by amphotropic PA317 cells, and cells of each human glioma were incubated with these particles in the presence of 8 μg/ml polybrene for 48 h. Transduced glioma cells were selected with G418 at an appropriate concentration, and these glioma cells were used for an assay of sensitivity to GCV. RSV-M mouse glioma cells transduced with MBP1.3/pNT230 (Fig. 1B) containing the 1.3-kb MBP promoter and the HTK gene served as a positive control for increased sensitivity to GCV. At 0.1 μM GCV, only 20% of RSV-M glioma cells survived, and almost no cells survived at a concentration of 1 μM or higher (high sensitivity to GCV) (Fig. 5). All wild-type human glioma cells which were not transduced with the HTK genes retained more than 90% viability even at 10 μM GCV (data not shown). More than 80% of ONS-75, U-138MG, or KNS-81 human glioma cells survived at 1 μM GCV (low sensitivity to GCV) (Fig. 5). In these low-sensitivity glioma cells containing MBP1.3/pNT230, more than 60% remained alive even at 10 μM GCV. About 50% and 30% of ONS-12, U-118MG, or KNS-60 human glioma cells survived concentrations of 1 μM and 10 μM GCV, respectively (moderate sensitivity group) (Fig. 5). U-87MG glioma cells showed low sensitivity at a lower concentration of GCV, but as the concentration

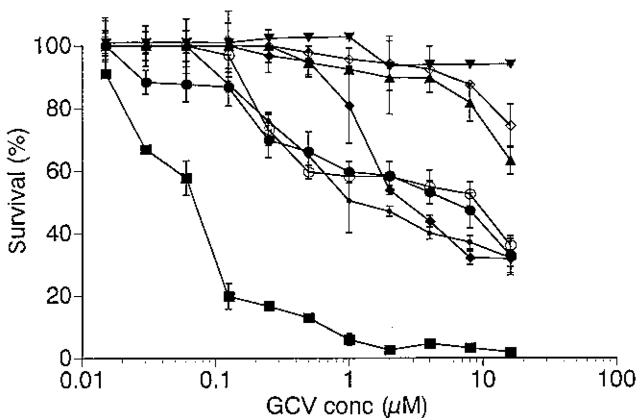

Fig. 5. Survival curves for glioma cells transduced with MBP1.3/pNT230 retroviruses and incubated with ganciclovir. Triplicate cultures (2×10^4/well) of glioma cells transduced with MBP1.3/pNT230 were incubated with various concentrations of ganciclovir (GCV) for 72 h, and then stained with crystal violet. RSV-M mouse glioma cells were sensitive to GCV concentrations of 0.1 *M* or higher after transduction with the retrovirus vector containing HTK gene under the control of the MBP promoter (■). ONS-12 (●), U-118MG (○), and KNS-60 (⬤) human glioma cells transduced with the same retroviruses (MBP1.3/pNT230) survived at rates of more than 50% at 1 *μM* and 30% at 10 *μM* GCV (moderately sensitive group). ONS-75 (▼), U-138MG (◇), and KNS-81 (▲) transduced with same retrovirus survived at rates of more than 80% at 1 *μM* and 60% at 10 *μM* GCV (weakly sensitive group). U-87MG (◆) human glioma cells transduced with the HTK gene-bearing retroviruses survived at rates of more than 80% at 1 *μM* and only 30% at 10 *μM* GCV (mixed sensitivity). Bars indicate the standard error of the mean.

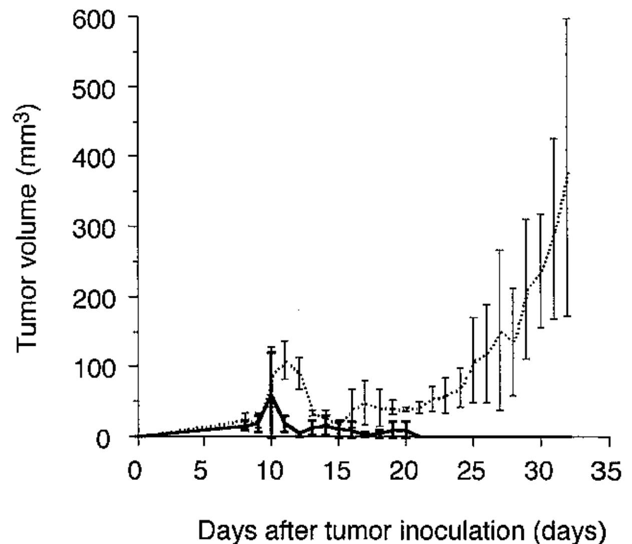

Fig. 6. Tumor volume arising from subcutaneously inoculated human glioma cells transduced with MBP1.3/pNT230 in nude mice. U-87MG human glioma cells (mixed sensitivity to ganciclovir (GCV)) were transduced with a retrovirus bearing the HTK gene regulated by an MBP promoter, and selected with G418 (0.5 mg/ml). These cells (1×10^7 cells/ 200 μl PBS) were inoculated subcutaneously into nude mice. These glioma cells were rejected after GCV treatment (50 mg/kg GCV was injected intraperitoneally once a day for 2 weeks) 22 days after tumor inoculation (solid line). On the other hand, these glioma cells formed a large mass without GCV treatment (dashed line). Bars indicate the standard error of the mean.

increased above 1 *μM*, sensitivity to GCV shifted from low to moderate (Fig. 5, closed diamonds).

These results showed that nucleoside analogs can kill glioma cells in which HTK gene expression is driven by the MBP promoter, but at least 30% of human glioma cells survive even a high concentration of GCV *in vitro*. This also indicates that it is important to develop an *in vitro* cytotoxicity test to check the sensitivity of human glioma cells to GCV after transduction with HTK gene-bearing retrovirus. However, since there is a difference in the sensitivity of HTK gene-transduced cells to GCV between *in vitro* and *in vivo* conditions, we investigated the *in vivo* sensitivity to GCV of human glioma cells transduced with the HTK gene. Some human glioma cells transduced with MBP promoter-HTK gene-bearing retroviruses were more sensitive to GCV *in vivo* than *in vitro*, partly because of the host immune reaction[25] (unpublished data). Human glioma cells transduced with HTK gene-bearing retroviruses were less sensitive to GCV *in vitro* than transduced RSV-M glioma cells (Fig. 5). Thus, the amount of intraperitoneal GCV injection was increased from 10 mg/kg to 50 mg/kg, because human glioma cells become sensitive at higher concentrations of GCV (Fig. 5). Since U-87MG showed relatively high MBP promoter activity, and because it consistently produces malignant tumors in nude mice, we used this human glioma cell line for an *in vivo* sensitivity assay to GCV. U-87MG cells transduced with MBP1.3/pNT230 were rejected after GCV treatment (Fig. 6, solid line), but the same cells formed a tumor mass without GCV treatment (Fig. 6, dashed line). Genetically unmodified U-87MG cells also produced a large mass in nude mice despite GCV treatment (data not shown). In U-118MG glioma cell lines, a similar tendency to that in U-87MG glioma cells was observed (data not shown). These results show that subcutaneously inoculated human glioma cells that are relatively insensitive to GCV *in vitro* can be rejected after intraperitoneal injection of GCV.

DISCUSSION

We have previously reported that a promoter inserted between two LTRs retains its tissue specificity even in the presence of a generally active enhancer located in the LTR,[9] and we used this promoter system to search for a glioma-specific promoter. Among several glial-specific promoters investigated, an MBP promoter has been shown to be most promising for glioma gene therapy, as indicated by specific and efficient transcription in mouse glioma cells.[7] We originally hypothesized that the most suitable promoter for regulating the toxic gene expression in glioma cells would be the GFAP promoter, because it is broadly expressed in astrocytes and glioma cells. However, the promoter activity of GFAP was lower than that of MBP in our experiments in mouse glioma cells. As GFAP is produced in mature glial cells, it may be unsuitable for controlling gene expression in undifferentiated malignant glioma cells. On the other hand, MBP is an oligodendrocyte marker which is also expressed in developing glial cells.[26] Certain gliomas have been shown to express O4 antigen, which is generally used as a marker for immature oligodendrocytes.[27] Several glioma cell lines used in the present work, especially RSV-M mouse glioma cells, were monoclonal antibody O4-positive (unpublished data) and therefore some glioma cells may share certain features with immature oligodendrocytes. However, we could not detect MBP-mRNA by northern blot analysis in RSV-M glioma cells (data not shown). Since MBP promoter activity is upregulated when oligodendrocytes begin to transcribe the MBP gene in several transgenic mouse systems,[28-30] the stability of the MBP-mRNA does not seem to play a crucial role in normal MBP biosynthesis. Therefore, it is not clear why the MBP promoter is so strong in glioma cells.

To identify *cis*-lying elements responsible for the efficient transcription initiation in glioma cells, we constructed two deletion mutants from the 5′ end. The shortest (256-bp) fragment showed promoter activity similar to that of the 1.3-kb fragment of the mouse MBP promoter region when we transduced MBP promoter-*lacZ* gene constructs in RSV-M glioma cells. MBP promoter was very active in this cell line even though MBP-mRNA was undetectable by northern analysis. The 256-bp fragment of the MBP promoter region showed the highest activity of any length of promoter region tested in NG108-15 cells.[31] In transgenic mice, the 256-bp MBP promoter region exhibited a peculiar expression pattern. This promoter region became active in oligodendrocytes only after they made axonal contact,[11, 24] while promoter regions longer than 1.3-kb were very active in oligodendrocytes even before myelination started.[12] Therefore, high promoter activity of the proximal MBP promoter region in certain glial cell lines, though not reflecting the *in vivo* situation, has importance for clinical application. We are currently mapping more precisely which *cis*-lying elements are important in directing strong expression in glioma cells. Until we pinpoint the *cis*-lying element(s), we reason that since the longest (1.3-kb) promoter fragment is known to be active in a variety of glioma cells, we will still use this 1.3-kb MBP promoter region for glioma treatment.

Before considering the use of the MBP promoter in clinical trials of malignant glioma gene therapy, we have to show that the effectiveness of the MBP promoter in human glioma cells is similar to that in murine glioma cells. After normalization with respect to the β-gal activity, the MBP promoter had the strongest activity among the glial-specific promoters in most human glioma cell lines. The activity of the GFAP promoter was stronger than that of the MBP promoter only in C6 rat glioma cells (Fig. 4B). Though this result is interesting given that C6 rat glioma cells produce GFAP, the MBP promoter had higher activity than the GFAP promoter in several GFAP-positive human glioma cells (KNS-81, KNS-89 and ONS-12). These results indicate that the MBP promoter is the best candidate among the glial-specific gene promoters tested here to control suicide gene expression in human glioma cells.

The results of our *in vitro* and *in vivo* assays of GCV cytotoxicity to human glioma cells transduced with the MBP1.3/pNT230 retrovirus vector (Figs. 5 and 6) suggested that human glioma-cell growth could be controlled by the combination of an MBP promoter-HTK gene and GCV, if the retroviral vector has a high enough infectious titer. Retrovirus-targeted gene therapy for malignant glioma using this MBP promoter may therefore prove to be a promising and relatively safe candidate for clinical trials.

ACKNOWLEDGMENTS

We thank Drs. T. Yoshimatsu, M. Yamada, T. Kagawa and K. Tamura for helpful discussions. This work was supported by a Grant-in-Aid for Scientific Research (08457363) from the Ministry of Education, Science, Sports, and Culture, and by grants from The Setsuro Fujii Memorial Foundation for Promotion of Fundamental Medical Research, and the Yamanouchi Foundation for Research on Metabolic Disorders.

(Received April 18, 1997/Accepted May 30, 1997)

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