In Situ Retroviral-mediated Gene Transfer for the Treatment of Brain Tumors in Rats

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ABSTRACT

Gene transfer with vectors derived from murine retroviruses is restricted to cells which are proliferating and synthesizing DNA at the time of infection. This suggests that retroviral-mediated gene transfer might permit targeting of gene integration into malignant cells in organs composed mainly of quiescent nonproliferating cells, such as in the brain. Accordingly, selective introduction of genes encoding for susceptibility to otherwise nontoxic drugs ("suicide" genes) into proliferating brain tumors may be used to treat this cancer. We investigated the efficacy and dynamics of in vivo transduction of growing brain tumors with the herpes simplex-thymidine kinase gene. Controls received vector producer and nonproducer NIH 3T3 cell lines containing the Escherichia coli lacZ (β-galactosidase) gene as well as nonproducer NIH 3T3 cells containing the thymidine kinase gene. The animals were treated to allow time for in situ transduction of the proliferating tumor cells with the herpes-thymidine kinase retroviral vector. The animals were treated with ganciclovir, 15 mg/kg i.p. twice a day for 14 days. Giolomas receiving an injection of 3-5 x 10⁶ thymidine kinase producer cells regressed completely in 23 of 30 rats given ganciclovir therapy, while 25 of 26 control rats developed large tumors. Intratumoral injection of a lower concentration of thymidine kinase vector producer cells (1.8 x 10⁶) resulted in a lower frequency of tumor regression (5 of 13 rats).

To estimate the efficiency of in vivo gene transfer, 9L brain tumors were given injections of 5 x 10⁶ β-galactosidase vector producer cells. S-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining revealed maximal staining of β-galactosidase within the tumor 7-14 days after injection of the vector producer cells. In vivo transduction rates in harvested tumors ranged from 10 to 70%. There was no evidence of transduction of the surrounding normal neural tissue. Occasional blood vessel endothelial cells within or adjacent to the tumor were observed to be S-bromo-4-chloro-3-indolyl-β-D-galactopyranoside positive. It is probable that destruction of this local vasculature with ganciclovir therapy also contributes to the efficacy of tumor regression.

Our results substantiate the feasibility of this approach for the treatment of malignant brain tumors in humans.

INTRODUCTION

Glioblastoma, the most common malignant primary brain tumor, is considered incurable. Multimodal approaches, such as surgery, radiation therapy, and chemotherapy, contribute little to the limited (9-12 months) median survival of patients (1).

The preferential infection and integration of retroviruses into dividing cells offer a unique mechanism for targeting gene transfer to the tumor with destruction of glioma cells while the contiguous, nondividing brain is protected (2). Several gene transfer systems have been described for the local treatment of cancer. These systems utilize ex vivo gene transfer with cytokine or major histocompatibility genes (3-6) to induce immune-mediated tumor regression. Another approach is the use of drug susceptibility ("suicide") genes (7-11) for selective destruction of the tumor. These gene transfer systems use retroviral vectors incapable of replication that are continually released by a genetically engineered producer cell line (12).

The HSVt gene is an example of a "suicide" or drug susceptibility gene. Cells transduced with the HSV gene become sensitive to the antiviral drug GCV, while nontransduced cells are unaffected (7-10). Ganciclovir is converted by HSVt to a nucleotide-like precursor that, following further phosphorylation, is incorporated into the DNA of dividing cells and leads to the arrest of DNA synthesis and cell death (7-10).

In an effort to develop in vivo gene transfer methods for the treatment of localized malignancy, we tested whether the direct injection of HSVt vector producer cells into growing rat brain could have an antitumor effect. We have recently reported our preliminary studies in which the in vivo transduction of brain tumors with the HSVt gene was successfully achieved in the rat brain tumor model. In that experiment, the injection of 3 x 10⁶ HSVt producer cells followed by high-dose ganciclovir treatment (300 mg/kg/day) induced regression of HSVt-transduced tumors in 1 of 14 treated animals compared to 100% tumor incidence in controls (13).

In the present report, we expanded our studies in the rat brain tumor model to investigate the dynamics of in vivo transduction as well as the dose response of ganciclovir and producer cell concentration on regression of transduced tumors.

We used the rat 9L gliosarcoma as a model of malignant brain tumors. 9L cells rapidly form an infiltrative tumor when injected into the brain and result in 100% mortality within 3 to 4 weeks (14). Initial in vitro studies compared the GCV sensitivity of the transduced rat 9L and the human glioblastoma cell line U251, confirming that human tumor cells are also sensitive to the HSVt/GCV system. In vivo studies evaluated the qualitative and quantitative dynamics of gene transfer by examining in situ transduction of brain tumors and normal brain in rats with the Escherichia coli reporter gene lacZ. Since the previous reports of the antitumor effect of HSVt/GCV used doses of GCV that are toxic to humans, dose response studies of tumor regression with GCV were performed in animals with brain tumors generated by inoculation of 9L cells pretreated with HSVt. Finally, the efficacy of tumor regression following in vivo tumor transduction with three concentrations of HSVt producer cells and GCV was assessed.

MATERIALS AND METHODS

Vectors and Cell Cultures. The β-galactosidase (G1BgSvN.29) and HSVt (G1TKeSvNa.90) vectors were generously provided by Genetic Therapy, Inc.
The GI backbone of these vectors is derived from the Moloney murine leukemia virus. The G1TkSvNa.90 vector contains the herpes simplex thymidine kinase gene just downstream of the 5’ long terminal repeat sequence and used this long terminal repeat sequence as its promoter. The SV40 early promoter serves as an internal promoter for the neomycin phosphotransferase gene. NeoR, which confers resistance to the neomycin analogue G418. In the β-galactosidase vector, the lacZ gene replaces the HSk gene. The HSk and β-galactosidase vectors are packaged by the amphotropic retroviral vector producer cell lines (PAT 2.4 and PAT317, respectively). Both producer cell lines were derived from NIH 3T3 cells. The preparation of G1BgSnV.29 used for these studies had a titer of 0.5–1.0 × 10^6 cfu/ml on NIH 3T3 cells. The G1TkSvNa.90 producer cell line generates a supernatant with a titer of 0.5 × 10^6 cfu/ml. Both cell lines were negative for replication-competent virus by sarcoma-positive, leukemia-negative assay.

The cloned vector producer cell lines were maintained in culture in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 2 mm l-glutamine (GIBCO BRL, Gaithersburg, MD), 50 units/ml penicillin (GIBCO BRL, Gaithersburg, MD), 50 μg/ml streptomycin (GIBCO BRL), and 2.5 μg/ml Fungizone (ICN Biomedicals, Inc., Costa Mesa, CA). The producer vector cells were grown in T-175 flasks. When used for in situ gene transfer, the medium was removed and the cells were rinsed with saline. The monolayer was then incubated at 20.5% trypsin-EDTA (GIBCO) for 5–10 min at 37°C. The cells were collected in HBSS (Biofluids Inc., Rockville, MD), washed twice, and resuspended in 0.5–1.0 × 10^6 cells/ml for infection.

In Vivo Sensitivity to Ganciclovir. The G1TkSvNa.90 vector was transferred in vitro into rat 9L gliosarcoma and human U251 glioblastoma cell lines using supernatant collected from confluent producer cell lines. The transduced cell lines were then selected in G418, 1.0 mg/ml (active drug, GIBCO), for 7 days. The sensitivity to GCV of these HSV-transduced, G418-selected cells was measured with a triitated thymidine incorporation assay. One × 10^6 cells were cultured in triplicate wells in increasing concentrations of GCV (Syntex Laboratories, Inc., Palo Alto, CA) for 24–48 h in flat-bottomed 96-well microtiter plates. Six h before harvest, 0.5 mCi tritiated thymidine (NEN) was added to each well. Data are expressed as mean cpm.

9L Brain Tumor Inoculation, Cell Injection, and GCV Administration. Fischer 344 rats weighing 230–350 g were anesthetized using i.p. ketamine (90 mg/kg; Fort Dodge Laboratories, Inc., Fort Dodge, IA) and xylazine (10 mg/kg; Mobay Corporation, Shawnee, KS) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Four × 10^4 syngeneic 9L gliosarcoma cells (14) in 5 μl Hanks’ balanced salt solution were injected into the deep white matter of the right cerebral hemisphere (depth of inoculation, 3.5 mm) using a 10-μl Hamilton syringe connected to the manipulating arm of the stereotaxic apparatus. Seven days later, the same stereotaxic coordinates were used to directly inject 1.8 × 10^6 (n = 13), 3.0 × 10^5 (n = 18), and 5 × 10^5 (n = 12) HSk producer line cells into the growing 9L tumor. All i.t. injections were given in a volume of 50 μl injected over 15 min. The needle was retracted over 5 min. Two rats received an i.t. injection of 50 μl of G1BgSnV.29 supernatant instead of cells. This volume contained approximately 2.5–5.0 × 10^5 vector particles. Seven days after the cell or supernatant injections, GCV was administered i.p. at 15 mg/kg twice daily (1 ml/injection) for 14 days. The rats were then sacrificed to quantitate antitumor effect. To determine the presence of residual tumor, a histological examination of the inoculation site (3–5 slices 10 μm apart) was performed in all animals.

Control rats received 3 × 10^5 G418-selected nonproducer NIH 3T3 fibroblasts transduced with the HSk (n = 6) or β-galactosidase (n = 6) genes. Additional controls received injections of the HSk vector producer cells (n = 8) or fibroblasts transduced with HSk (n = 6) followed by i.p. saline injections (1 ml, twice daily) without subsequent GCV treatment.

Initial experiments were complicated by infections probably secondary to GCV-induced bone marrow suppression. The use of sterile bedding and food and the addition of tetracycline to the drinking water (approximately 75 mg/kg) diminished this problem. In addition, all rats received demethylamine (TechAmerica, Kansas City, MO), 0.5 mg/kg/20 ml (estimated daily water intake), in the drinking water starting on the day of producer cell injection for 10 days.

GCV Dose Optimization. For GCV dose optimization studies, rats were intracerebrally inoculated with 4 × 10^6 HSk-transduced 9L cells. Seven days after inoculation, GCV was administered i.p. at 5, 10, or 15 mg/kg twice daily (1 ml/injection) for 7 days (n = 10 rats in each group). Control rats (n = 10) received i.p. saline injections (1 ml, twice daily). All rats were anesthetized and then sacrificed following 7 days of GCV therapy by intracardiac perfusion with heparinized saline (100 units/100 ml) followed by 4% formaldehyde in saline (300 ml) for in situ fixation of the tumors. The brains were then removed and the tumors were dissected carefully from the frontal lobe and weighed.

Dynamics and Quantification of Gene Transfer. Rats studied with the β-galactosidase gene received 3 × 10^6 G1BgSnV.29 producer cells (n = 24) or nonproducer fibroblasts that had been transduced with the β-galactosidase gene (nonvector producer) and selected in G418 for 7 days (n = 22) into 9L tumors or normal brain. Injections were performed as described above. Rats were then sacrificed on days 3, 7, 10, 14, and 17 as described above. Brain was removed, sectioned, and stained with X-Gal (see below). The 9L cells used for tumor inoculation were completely negative for β-galactosidase activity while 100% of the β-galactosidase vector producer and nonproducer cells were X-Gal positive.

For quantitative estimation of tumor transduction, 9L tumors were harvested 7, 9, 11, and 13 days after i.t. injection of the β-galactosidase vector producer, nonproducer cells, or supernatant containing the free β-galactosidase retroviral vector. The tumors were minced and dissociated by mechanical stirring for 2 h in a sterile glass bottle containing HBSS with 0.01% hyaluronidase type V (1500 units/g), 0.1% collagenase type IV (163–230 units/g), and 0.002% DNase type I (100 units/mg; Sigma Chemical Company, St. Louis, MO). The cell suspension was then filtered through nylon mesh and washed 3 times in HBSS. The cells were counted and either stained with X-Gal or placed in culture in RPMI 1640 (GIBCO) with 10% fetal bovine serum (HyClone), 2 mm l-glutamine (GIBCO), 50 units/ml penicillin (GIBCO), 50 μg/ml streptomycin (GIBCO), and 2.5 μg/ml Fungizone (ICN Biomedicals) at 1 × 10^6 cells/well in 24-well plates (Costar). When the cells began to proliferate, they were selected in 1.0 mg/ml G418 (GIBCO) for 7 days. At the end of the selection period, the cells were washed and placed in fresh medium for 4 days before staining with X-Gal. Data are expressed as the percentage of X-Gal-positive cells.

Histochemical Staining. β-Galactosidase expression was detected using an X-Gal histochemical stain (15). Staining the brain with X-Gal turns β-galactosidase-expressing cells blue when an indolyl is liberated from X-Gal by the action of β-galactosidase and subsequent oxidation and self-coupling forms an indigo blue derivative. The vector-containing cells can thus be discriminated from unmodified cells and enumerated with light microscopy. Cultured cells were stained with X-Gal only. The histological tissue sections were counterstained with hematoxylin and eosin following X-Gal staining or with hematoxylin and eosin alone.

RESULTS

In Vitro Sensitivity of 9L and U251 Cells to GCV. Rat 9L glioma cells and the human U251 glioblastoma cells were transduced in vitro with the HSk gene by exposure to supernatant from HSk producer cells cultures containing replication-incompetent vector particles (0.5–1.0 × 10^6 cfu/ml). To achieve an enriched population of transduced cells, the tumor cells were then selected in G418 (1.0 mg/ml) for 7 days. After G418 selection, cells were exposed to increasing concentrations of GCV for 24 to 48 h and DNA synthesis was evaluated by triitated thymidine incorporation. Both the 9L (Fig. 1A) and U251 (Fig. 1B) cell lines were sensitive to low concentrations of GCV (0.5–5 mg/ml) and cell toxicity increased with duration of GCV exposure (9L). Triitated thymidine incorporation by the control tumor cells was not significantly decreased after GCV exposure; i.e., a susceptibility to GCV was acquired by the transduced cells. These findings confirm that retroviral vectors containing the HSk gene can effectively transduce rat and human brain tumor cells and that the tumor cells express functional HSk.

Dynamics of in Vivo Transduction with the β-Galactosidase Gene. In vivo transduction of brain tumors with the β-galactosidase gene allowed us to determine the efficiency of in situ transduction and relative accumulation of β-galactosidase by the tumor cells.
After localized deposit of the β-galactosidase producer cells at the site of the previously implanted tumor, tumor cells containing blue-staining granules were used as an indication of gene transfer and expression.

There was a clear delineation between the transduced tumor cells and normal brain, which completely lacked X-Gal-positive cells (Fig. 2A). The accumulation of the β-galactosidase enzyme in tumor cells increased with time as was apparent by the progressive increasing intensity of the blue stain in each transduced cell. Maximal expression (or accumulation) of β-galactosidase was reached between 7 and 14 days after injection of the β-galactosidase vector producer cells (Fig. 2, B-D). The typical appearance of the tumor 14 days after vector producer cell injection was one of scattered colonies of X-Gal-positive tumor cells, composed of transduced tumor cells from the injected producer cells as well as the progeny of these cells. Nontransduced tumor was seen between the scattered X-Gal-stained colonies (Fig. 2A).

In the non-tumor-bearing hemisphere injected with β-galactosidase producer or nonproducer cells, X-Gal-positive cells were detected in diminishing numbers for up to 14 days, by which time they had all disappeared from the injection site. This interval probably represents the life span of these xenogeneic murine cells in the brain. No local inflammatory cell infiltrate was seen when dexamethasone was given.

Transduction of occasional endothelial cells, within or adjacent to the tumor, was observed in most rats (Fig. 3). This was less evident when β-galactosidase producer cells were injected into the non-tumor-bearing hemisphere. Transduction of normal brain (neurons and glial cells) was not detected in the tissue surrounding the tumor or in the β-galactosidase producer cell-injected, non-tumor-bearing hemisphere. As expected, animals that received the nonproducer control cells did not show evidence of transduction of tumor cells or normal brain.

Quantitative assessment of the efficiency of in situ tumor transduction was estimated in tumors removed 7, 9, 11, and 13 days after injection of β-galactosidase producer cells, NIH 3T3 cells that had been transduced in vitro with the β-galactosidase gene, or retroviral-vector supernatant collected from a confluent tissue culture of β-galactosidase vector producer cells. Measurement of the rate of transduction 7–13 days after injection of the cells or supernatant (Table 1) showed that after incubation with G418 to select for cells expressing NeoR, greater than 95% tumor cells stained positive with X-Gal. All cells derived from control animals died in G418.

**Dose Optimization of GCV.** Since the high-dose GCV treatment used in preliminary studies (300 mg/kg/day) is highly toxic to humans, the efficacy of the relevant human dose (10 mg/kg/day) and two higher doses, 20 mg/kg and 30 mg/kg/day, was evaluated (Fig. 4). In rats inoculated with HSik-transduced 9L tumor cells all three doses induced regression of tumors when compared to the control group (analysis of variance, P < 0.001). No difference in mean tumor weights was observed among the 3 GCV doses. Histological examination after 7 days of treatment showed some residual viable tumor in each group. Therefore, we used a 14-day GCV treatment course for our in vivo tumor transduction experiments.

**Tumor Regression with in Vivo HSik Transduction and GCV.** Based on the results of the GCV dose-optimization study, a dose of 15 mg/kg per dose was used in i.p. injections twice a day (30 mg/kg/day). Table 2 summarizes the results obtained from treatment of rats carrying 9L brain tumors injected with HSik producer cells or control cells followed by GCV administration. Antitumor effect was determined by examining the rat brains 28 days after inoculation of the tumor (following 14 days of GCV therapy). The injection of 1.8 × 10⁶ HSik producer cells into the tumor resulted in complete tumor regression in 5 of 13 rats (38%), while injection of a greater number of cells led to tumor regression in 13 of 18 (72%) and 10 of 12 rats (83%) when 3 × 10⁶ and 5 × 10⁶ HSik producer cells, respectively, were injected. Histological examination of the tumor inoculation site in rats with complete tumor regression revealed focal necrosis and hemorrhage with reactive gliosis (Fig. 5, A and B). Residual tumors in the treated rats were smaller than tumors in the control animals and contained multiple hemorrhagic necrotic foci (Fig. 5, C versus D). In contrast, 25 of 26 control rats developed progressive brain tumors (Fig. 5D). The one control rat with no evidence of tumor had received i.t. HSik-transduced (nonproducer cells) NIH 3T3 cells and GCV for 14 days.

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Fig. 1. A, sensitivity of 9L glioma cells (measured by initiated thymidine incorporation) to different concentrations of GCV for 24 (○) or 48 h (●). , , nontransduced cells; —, cells transduced carrying the HSik gene. B, sensitivity of human U251 glioma cells (measured by initiated thymidine incorporation) exposed to different concentrations of GCV for 48 h. , , nontransduced cells; —, cells transduced with the HSik gene.
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Fig. 2. In vivo transduction of intracerebral 9L glioma. A, interface between the tumor and surrounding brain 14 days after injection of β-galactosidase producer cells. Note sharp delineation between the β-galactosidase-expressing transduced tumor and nontransduced normal brain (X-Gal stain and H&E counterstain, × 100). B-D, expression of β-galactosidase within tumor cells at day 3 (B), 7 (C), and 14 (D) after i.t. injection of the β-galactosidase vector producer cells. (X-Gal stain and H&E counterstain, × 1000).

Ten rats died during GCV therapy and are not included in the data presented in Table 2. Three deaths were attributed to i.p. hemorrhage (a result of repeated i.p. injections or GCV-induced thrombocytopenia) and the rest were attributed to infectious complications. In all these rats the brains were removed and inspected for the presence of tumor. Five of the animals were tumor free (all treated with $5 \times 10^6$ HSik producer cells). In the remaining 5, residual tumor was found (3 in rats receiving $1.8 \times 10^6$ HSik producer cells and 2 in rats treated with $3 \times 10^6$ HSik producer cells).

DISCUSSION

The localized and nonmetastatic nature of malignant primary brain tumors renders them optimal candidates for selective local therapy such as in situ transfer of a drug susceptibility gene. This approach enables preferential tumor kill while avoiding toxicity to the neighboring nondividing neural tissue. Our results confirm the feasibility of in situ retroviral-mediated gene transfer into malignant brain tumors and demonstrate tumor regression when HSik transduction is coupled with GCV therapy. Similar vectors carrying the HSik gene have been described in recent reports for the treatment of sarcoma, lymphoma, and glioma cells in tissue cultures (9–10). In vivo inhibition of tumor growth was also achieved following GCV when the HSik gene had been transduced into the tumor cells in vitro before inoculation of the tumor (7, 9–10). The HSik gene transfer methods described in these other reports differ from our approach in which in vivo injection of the vector producer cells into an established brain tumor produced successful gene transfer and integration (13). In addition, the inhibition of tumor growth observed in the prior reports was achieved with GCV administration at doses toxic to humans. We show that GCV can produce a significant tumor response even when it is given at a dose similar to that safely given to patients for treatment of ocular viral infections (16). However, it appears that the drug must be given for a longer duration (14 versus 7 days) to achieve complete tumor eradication. The optimal duration of GCV therapy is unknown. In our optimization studies viable tumor cells were still present after 7 days of GCV treatment. Accordingly, the duration of GCV therapy was empirically extended to 14 days in an attempt to overcome partial responses. The timing for initiation of and the duration of GCV therapy were also based on the assessment of gene transfer dynamics using the β-galactosidase gene, which showed that maximal gene expression (or accumulation of the enzyme) and transduction occur between 7 and 14 days after i.t. injection of the β-galactosidase producer cells.

The ratio of the injected vector producer cells to tumor cells seems to have an important role in achieving in vivo tumor transduction and regression. Nearly doubling the number of injected vector producer cells in our brain tumor model led to doubling of the incidence of complete tumor regression (38% versus 72% for injections of $1.8 \times 10^6$ and $3 \times 10^6$ cells, respectively). Despite the in vivo transduction of an estimated 10–70% of the tumor cells, as suggested by the β-galactosidase gene transfer studies, complete tumor eradication occurred in most of the treated rats. This phenomenon is probably due to a "bystander" effect associated uniquely with the HSik/GCV system.
Table 1  Quantification of gene transfer by X-Gal staining of 9L tumor cells

<table>
<thead>
<tr>
<th>Injected cells or supernatant</th>
<th>No. of days after in situ treatment</th>
<th>X-Gal-positive cells (%)</th>
<th>X-Gal-positive cells (%)</th>
<th>X-Gal-positive cells (%)</th>
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<td></td>
<td>(direct stain)</td>
<td>(cultured cells)</td>
<td>(G418-selected)</td>
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<tr>
<td>GIBgSvN.29 producer cells (n = 6)</td>
<td>7</td>
<td>NT</td>
<td>55</td>
<td>95</td>
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<td>70</td>
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<td>90</td>
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<tr>
<td></td>
<td>7</td>
<td>NT</td>
<td>0</td>
<td>Alldead</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>Alldead</td>
</tr>
<tr>
<td>Direct GIBgSvN.29 supernatant (n = 2)</td>
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<td>0</td>
<td>Alldead</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>1</td>
<td>Alldead</td>
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<tr>
<td>9L tumor (n = 2)</td>
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* Results are expressed as percentage of X-Gal-positive cells.

Table 2  Fraction of animals with complete tumor regression 21 days after treatment

<table>
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<tr>
<th>Treatment group</th>
<th>GCV (15 mg/kg)</th>
<th>No. of injected cells</th>
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<tr>
<td></td>
<td>TK producer*</td>
<td>GCV 5/13 (38)*</td>
</tr>
<tr>
<td></td>
<td>TK producer*</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td></td>
<td>3T3-BAG*</td>
<td>GCV 0/6 (0)</td>
</tr>
<tr>
<td></td>
<td>3T3-TK*</td>
<td>GCV 1/6 (17)</td>
</tr>
<tr>
<td></td>
<td>No GCV</td>
<td>0/6 (0)</td>
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* NIH 3T3 cells transduced with HStk gene.

Fig. 4. Mean tumor weights (bars, mg ± SD) of HStk-transduced 9L brain tumors harvested after 7 days of GCV therapy at 10, 20, and 30 mg/kg/day, compared to tumor weights of nontreated rats.

(13). Original demonstration of bystander effect was shown when mixtures of HStk-transduced tumors and wild-type tumors were inoculated s.c. in mice and subsequently treated with GCV. In animals bearing tumors with as few as 10% HStk-expressing cells, complete tumor regression was shown in over one-half the animals (13). We demonstrated the bystander effect in cultures of 9L and human U251 glioma cells where a mixture of 10% HStk-transduced cells with 90% nontransduced cells led to significant (>50%) reduction in cell proliferation (measured by tritiated thymidine incorporation) after exposure to low concentrations of GCV (data not shown). This bystander effect may also be responsible for the tumor regression observed in one of our control rats which received an i.t. injection of HStk-transduced NIH 3T3 cells and GCV. Direct injection of supernatant containing the GIBgSvN.29 retroviral vector did not lead to detectable transduction of any tumor cells, probably due to the short half-life (hours) of free vector and possible inactivation by serum complement. The residual tumor found in some of our HStk vector producer cell-treated rats may indicate insufficient tumor transduction, inaccurate injection of vector producer cells, or a variability in gene expression, with reduced tumor response to GCV.

The only evidence of gene transfer into nontumoral tissue was an occasional endothelial cell transduced with the β-galactosidase gene. These cells were all within, or immediately adjacent to, the tumor. The most mitotically active endothelial cells in the area of the tumor are likely to be those responding to angiogenesis factors released by the tumor. Transduction with the HStk gene and elimination of these transduced endothelial cells with GCV therapy may result in ischemia of the tumor mass and thereby further contribute to tumor eradication.

The principal technical limitation of this approach is reflected in the variable efficiency of gene transfer into tumors. Transduction efficacy seemed to be higher in our brain tumor model as compared to another similar study where in situ transfer of the β-galactosidase gene resulted in transduction of about 10% of the tumor cells (17). Differences in the vectors, tumor model, and expression or accumulation of the gene may have accounted for the different transduction rate. Variability of transduction was seen in our animals by the β-galactosidase gene transfer experiments in which transduction rates in tumors varied from 10 to 70% and may account for the absent or partial response to GCV of some of the tumors injected with HStk producer cells. Such variability probably results from a combination of uneven distribution of vector producer cells throughout the tumor, uneven cycling of tumor cells, and unequal drug delivery to the tumor. Loss of expression of HStk in successfully transduced cells may also underlie treatment failures although all recovered G418-selected 9L tumor cells were completely destroyed when exposed to GCV over 7 days. In addition, the HStk producer line has also remained 100% sensitive to GCV after months in culture (data not shown). This may change in vivo because even when 95–99% of inoculated 9L tumor cells were HStk positive prior to inoculation for our GCV optimization...
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Fig. 5. A. HStk vector producer-injected 9L brain tumor after 14 days of GCV therapy into a 14-day-old tumor. Note the absence of tumor and the residual necrotic areas at the inoculation site (long arrow). The needle track leading into the inoculation and injection site is apparent (short arrow). B. Diffuse necrosis and hemorrhage seen at site of tumor inoculation and HStk vector producer cell injection 10 days after initiation of GCV therapy. (H&E. × 400). C. Partial regression of a HStk vector producer-injected 9L brain tumor after 14 days of GCV therapy (4 weeks tumor age). Note the small tumor size with focal areas of hemorrhage and necrosis within the tumor (arrow). D. HStk vector producer cell-injected brain tumor without GCV therapy (4 weeks tumor age). Note large tumor growing out from the inoculation site (arrow).

studies, 7 days of GCV treatment did not lead to 100% tumor kill. This phenomenon was also observed in other reports evaluating the HStk/GCV system (9, 10). Accordingly, optimal timing of the initiation and duration of GCV treatment may not yet be determined since the transduction dynamics and expression could vary significantly among tumors and may depend on the efficiency of drug delivery and vascular supply of the tumor. Optimal response may require that the therapy be tailored for individual tumors according to their differing biological characteristics, such as growth rate or mitotic index. Modification of the vector producer cell lines for enhanced vector production and prolonged survival in vivo may enhance the efficacy of this approach. Improved in vivo gene transfer may be achieved by designing autologous packaging cell lines that would enable repeated injections of the vector producer cells without provoking an immune response. Finally, the use of replication-competent retroviruses may prove to be the most effective and efficient vehicle for selective gene transfer to tumor allowing a horizontal spread of the infection into essentially all tumor cells. The feasibility and risks involved with such approaches are yet to be evaluated.

This report confirms our preliminary data indicating that gene transfer can be achieved by in situ injection of retroviral vector producer cells into established brain tumors. The results from our studies of the dynamics of transduction and the optimization of GCV dose enabled us to develop an animal model for the successful use of in vivo gene transfer for the elimination of malignant brain tumors using the HStk gene to confer selective tumor sensitivity to GCV. The efficacy of this approach should be evaluated in human cancer patients as the initial step of direct, in vivo, therapeutic manipulation of the tumor genome.

REFERENCES
In Situ Retroviral-mediated Gene Transfer for the Treatment of Brain Tumors in Rats

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