Patients with recurrent malignant gliomas usually survive for less than 1 year. Reoperation is palliative, and chemotherapy, which is hampered by barriers to drug delivery and both neurological and systemic toxicities, produces responses in fewer than 30% of recipients. Gene therapy techniques that permit more selective targeting of tumor cells within the brain offer hope for both improved delivery and less toxicity.

The gene therapy strategy of selectively activating the thymidine analog GCV within tumor cells by retroviral vectors bearing the thymidine kinase (tk) gene has produced dramatic remissions of malignant gliomas in animal models. In human trials, although the technique has been deemed safe, little antitumor effect has been demonstrated. To evaluate the basis of this inefficacy in human gliomas, the authors conducted a gene-marking trial involving neuropathological and biochemical studies of treated tumor specimens.

Methods. Five patients with malignant recurrent gliomas underwent stereotactic biopsy sampling and intratumoral implantation procedures with three aliquots of $10^6$ vector-producing cells (VPCs) in columns. After 5 days, the tumor was resected and the tumor bed reimplanted with VPCs, and a course of GCV was given. Patients received clinical and radiological follow up for 6 months. Tumor specimens were analyzed neuropathologically and for tk gene expression by anti-TK immunohistochemistry and TK enzymatic activity.

Four patients tolerated the treatment well but experienced tumor progression. The other developed an abscess after the second operation and died. Increased TK enzymatic activity was demonstrated in the one tumor specimen analyzed. Immunohistochemical evidence of tk gene expression was limited to VPCs. Transduction of tumor cells was not seen. Viable tumor cells were seen near VPCs containing TK. The lymphocytic immune response was mild.

Conclusions. Except for the risk of infection inherent in reoperation, this tk–GCV paradigm was both feasible and safe. Pathological studies indicated that limited dissemination of VPCs and vector from the infusion site and failure to transduce tumor cells with the tk gene are major barriers to efficacy.

Key Words • gene therapy • brain neoplasm • ganciclovir • glioblastoma multiforme

Abbreviations used in this paper: GCV = ganciclovir; GFAP = glial fibrillary acidic protein; HSV-tk = herpes simplex virus thymidine kinase; MAb = monoclonal antibody; MR = magnetic resonance; mRNA = messenger RNA; VPCs = vector-producing cells.
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rats by using rodent and human glioma cells transduced in vitro with the tk gene.6,15,16 Because retrovirus injected directly into a tumor results in only very low levels of transduction, VPCs capable of continuously secreting retroviral vectors in situ were developed for in vitro transduction. Treatment of either subcutaneous or intracerebral gliomas in rodents by using GCV following either cografting or intratumoral inoculation with VPCs produced tumor regression and improved animal survival.1,5,15,17

The safety of intracerebral injection of VPCs has been demonstrated. In rodent glioma models, normal brain tissue is not altered adjacent to transduced tumor, the retroviral vector is not disseminated either intracerebrally or systemically, and systemic spread of VPCs following intracerebral injection is limited to lymphoproliferative organs (lung, spleen, liver, intestine), without harmful sequelae. In nonhuman primates, intracerebral injection of VPCs and subsequent treatment by using GCV produced no systemic, neurological, or radiographically detectable toxicity. Histological analysis showed only mild gliosis at the injection site.5,14

The first human trial of tk and GCV for brain tumors involved 12 patients with malignant gliomas and three with metastases. Two patients suffered intratumoral hemorrhage and neurological deficits after biopsy sampling and VPC injections. Five lesions in four patients exhibited either a complete (two lesions) or partial (three lesions) response on radiological studies.13 In tumor resected in two patients at 7 days posttreatment, tk transcripts were identified in surviving VPCs but in fewer than 0.2% of neighboring tumor cells on in situ hybridization. Very little immune response was seen. To address the questions of the safety of the injections, the rate of tumor cell transduction, the amplitude of the immune response, and the degree of antitumor effect of the tk–GCV paradigm, our study combined gene marking and a therapeutic trial. Two trials of intratumoral VPC implantation were separated by an intermediate harvest of infused tumor for neuropathological study as well as for assays of TK protein, TK enzymatic activity, and immune infiltrates before long-term clinical and radiographic follow up.

Clinical Material and Methods

Clinical Protocol

Conventional therapy for suspected recurrent glioma combines stereotactic biopsy sampling for confirmation of malignant regrowth with subsequent craniotomy for tumor resection.8 We adhered to this treatment plan, as approved by our institutional review board, in applying the tk–GCV paradigm. Eligible patients maintained a Karnofsky Performance Scale score of at least 70, were at least 18 years old, and met MR imaging and positron emission tomography criteria for regrowth of malignant glioma following biopsy procedures or resection and fractionated irradiation. We excluded patients who were pregnant, experiencing rapid neurological deterioration or infection, or who were afflicted by inadequate hematological, renal, or cardiopulmonary reserve.

Three regions of tumor seen on MR images were targeted for stereotactic biopsy procedures and VPC injection: contrast-enhancing solid tumor, the interface of contrast-enhancing tumor and nonenhancing tumor-infiltrated brain, and nonenhancing tumor-infiltrated brain. After biopsy sampling confirmed malignant tumor, 10-μl aliquots of 10⁸ VPCs were infused as 20-mm-long columns at each of the three sites (Figs. 1 and 2). A novel mechanized stereotactic infusion apparatus was used to infuse cells at 1 μl/minute and the injection needle was withdrawn at a rate of 2 mm/minute.12
Five days later, between 1 hour and 2 hours before tumor resection, a single dose (5 mg/kg) of GCV was administered intravenously. At craniotomy, the cortical entry sites of prior infusions were identified and marked with methylene blue. All three infusion tracks were included in the en bloc harvest of the tumor specimen. The specimens were further marked to identify track orientation and then divided for histopathological and pharmacological analyses. The tumor bed was then injected with 10 to 20 aliquots of a 10-μL vehicle containing either $5 \times 10^3$ or $5 \times 10^6$ VPCs in columns placed orthogonally to the resection margin at the center of 100-mm² squares. Beginning on the 5th postoperative day, a 2-week course of intravenous GCV (5 mg/kg every 12 hours) was given. Doses of steroid medications were tapered from a perioperative dose of 16 mg per day to nil as permitted by the clinical course.

Patients were examined clinically, hematologically, and radiographically at the following times: preoperatively; 5 days after biopsy sampling and infusion; and 1 day, 3 weeks, and 3 months after craniotomy. Clinical assessments included full neurological and general physical examinations, hematological, renal, and hepatic profiles, MR imaging without and with contrast, and positron emission tomography scans.

**Vector Structure and VPCs**

The VPCs, CRIP-MFG-S-TK, were used for Phase I study. The vector contains the $5'$ and $3'$ MoMuLV long-terminal repeats to drive transcription of the full-length and subgenomic RNA of HSV-1 tk, which is processed using the normal $5'$ and $3'$ splice sites of MoMuLV. The retroviral psi packaging sequence is retained. The structural gene for HSV-1 tk was cloned between the HindIII and BglII sites of MFG-S, to give rise to MFG-S-HSV-TK. Viral vector particles are produced by psi-CRIP packaging cells that have been stably transfected with MFG-S-HSV-TK. The psi-CRIP packaging cells were derived from the murine NIH-3T3 fibroblast cells by separate insertion of sequences encoding the viral packaging protein genes env and gag-pol.

The VPCs were stored in liquid nitrogen. On the day of treatment, individual vials were thawed, combined, rinsed thrice in cold buffered saline, counted, and checked for viability by trypan blue exclusion. Viability was consistently in excess of 90%.

**Histological and Immunohistochemical Studies**

The resection specimens were oriented by the neurosurgeon, and the infusion needle’s cortical entry sites were identified as punctate hemorrhagic foci. After photography, the specimens were cut perpendicular to the needle tracks, and the serial sections were examined grossly to identify the infusion paths. Representative portions of one section were frozen for subsequent pharmacological studies. The entire remaining length of each needle track and surrounding parenchyma was blocked sequentially for histological examination. Orientation was marked using colored inks. Representative portions of the distant tumor and infiltrated brain were also sampled. The sections were fixed in 10% formalin, processed, and embedded in paraffin for histological and immunohistochemical studies.

All formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin and examined microscopically by one of the authors (D.L.). For immunohistochemical studies, sections were cut onto charged slides, deparaffinized, and rehydrated. During rehydration, sections were immersed in 0.5% hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase activity. For MB-1 and T- and B-cell markers, sections were then microwaved in a sodium citrate buffer at high power for 15 minutes. Following rinsing in phosphate-buffered saline at pH 7.4, the sections were blocked with the appropriate normal serum (normal goat or normal horse serum) at 10% concentration for 30 minutes. The sections were then incubated with primary antibody overnight at 4°C.

The primary antibodies and dilutions in phosphate-buffered saline were as follows: TK-polyclonal antisera at 1:1000; GFAP MAb at 1:250; CD20/L26 MAb (B-cell marker) at 1:100; CD3 MAb (T cell) at 1:150; and MB-1 at 1:75. Sections were then treated with a biotinylated secondary antibody (horse anti–mouse or goat anti–rabbit), followed by the avidin–biotin complex kit. Diaminobenzidine was used as a chromogen, producing a dark brown reaction product, and the tissue was counterstained with either methyl green (for nuclear products) or hematoxylin (for cytoplasmic products). Intracerebral rat tumors of 9L gliosarcoma cells infected with TK-producing retrovirus served as positive controls for TK immunohistochemistry; human tonsil tissue provided positive controls for MB-1 as well as T- and B-cell markers; reactive astrocytosis adjacent to a human cerebral infarct was used as a positive control for GFAP staining. Negative controls included omission of the primary antibodies as well as staining of irrelevant tissues. To quantify the percentage of cell staining for TK and MB-1, cell counting was performed on an image analysis system, the components of which are listed in Sources of Supplies and Equipment.

**Quantification of TK Enzymatic Activity**

Tissues were shipped on dry ice and stored at −90°C. Tissue samples were homogenized in 0.1 M phosphate buffer at pH 7.8. The homogenates were centrifuged at 40,000 G and the supernatants were used to measure TK activity. The assay (in 0.1 M sodium phosphate buffer, pH 7.8) contained 10 mM adenosine 5'-triphosphate, 10 mM MgCl₂, 0.5 to 0.6 mg protein, and 0.5 μCi [³H]-labeled thymidine (14 mCi/mmol) or [³C]-labeled thymidine (14 mCi/mmol). After incubation at 37°C for 60 minutes, activity was stopped by the addition of perchloric acid. The acid-soluble supernatant was then analyzed for phosphorylated nucleotides by reversed-phase ion-pair high-performance liquid chromatography by collecting the effluent in 40 1-ml fractions. Radioactivity was determined by scintillation counting, and protein was measured using the microbichromic acid assay. The kinase activity was calculated by subtracting the percentage of total radioactivity in fractions obtained from an unincubated sample from that of the corresponding sample incubated at 37°C. The rat 9L gliosarcoma cell line, stably transfected with TK, served as a positive control.

Under these conditions, GCV is phosphorylated only by the HSV-derived TK enzyme present in VPCs and any transduced tumor cell. Thymidine is phosphorylated by
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Characteristics of the five patients with GBM who received VPC infusions*

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yrs), Sex</th>
<th>Tumor Histology &amp; Location</th>
<th>Previous Treatment</th>
<th>VPC Injections</th>
<th>GCV Doses</th>
<th>Treatment and outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45, F</td>
<td>initial &amp; recurrent GBM, rt frontal</td>
<td>gross-total resection, radiation (60 + 15 Gy)</td>
<td>10 6 1 28</td>
<td>0 28</td>
<td>disease progression, alive at 18 mos</td>
</tr>
<tr>
<td>2</td>
<td>42, M</td>
<td>initial oligodendroglioma, recurrent GBM, lt frontal</td>
<td>near-total resection, radiation (60 Gy), chemo: cisplatin 5, carboplatin 18 cycles</td>
<td>2 3 10^6</td>
<td>12 @ 5 x 10^6</td>
<td>1 28</td>
</tr>
<tr>
<td>3</td>
<td>30, M</td>
<td>initial oligoastrocytoma, recurrent GBM, rt temporal</td>
<td>partial resection, radiation (54 Gy), chemo: PCV 4 cycles</td>
<td>3 3 10^6</td>
<td>20 @ 5 x 10^6</td>
<td>1 28</td>
</tr>
<tr>
<td>4</td>
<td>79, M</td>
<td>initial &amp; recurrent GBM, lt parietal</td>
<td>gross-total resection, radiation (60 Gy), chemo: PCV 2 cycles</td>
<td>4 3 10^6</td>
<td>15 @ 5 x 10^6</td>
<td>1 28</td>
</tr>
<tr>
<td>5</td>
<td>41, F</td>
<td>initial &amp; recurrent GBM, lt frontal</td>
<td>gross-total resection, radiation (60 Gy), chemo: marimastat/placebo</td>
<td>5 3 10^6</td>
<td>0</td>
<td>0 0</td>
</tr>
</tbody>
</table>

* There were no complications except in the patient in Case 4, who suffered from a cerebral abscess and renal failure.

Both the HSV-derived TK and the endogenous mammalian TK. Endogenous mammalian TK enzyme should be expressed almost exclusively in tumor cells in the G1/S phase but not in other tumor cells or quiescent normal cells.

Sources of Supplies and Equipment

The VPCs were supplied by the Somatix Therapy Corp., Alameda, CA, under Food and Drug Administration–approved agreement no. IND BB 5229. The Probe-On slides used for immunohistochemical studies were obtained from Fischer Scientific, Fair Lawn, NJ. The TK-polyclonal antisera were kindly provided by Dr. W. Summers, Yale University, New Haven, CT. The GFAP, CD20/L26, and CD3 MAb's were purchased from Dako Corp., Carpinteria, CA. The MIB-1 was acquired from Amac, Inc., Westbrook, ME. The avidin–biotin complex kit (ABC Elite) was obtained from Vector Laboratories, Burlingame, CA. The image analysis system consisted of the following components: a BX60 microscope purchased from Olympus Optical Co., Tokyo, Japan; a 3CCD color video camera acquired from Sony Corp., Tokyo, Japan; a PowerTower Pro 200 computer obtained from Power Computing, Austin, TX; and Image Pro Plus software purchased from Media Cybernetics, Silver Spring, MD.

Results

Clinical Outcome

Of 20 patients screened, eight met eligibility criteria and six consented to the study. In one patient only radiation necrosis was demonstrated on all three biopsy samples and thus the individual did not receive VPC infusions. Of the five patients who received the first VPC infusions (Table 1), only the second and third completed the entire protocol (the first patient did not receive the single dose of GCV before craniotomy). Midway through the 2 weeks of GCV therapy the fourth patient developed an intracranial abscess and subsequently died. The fifth patient underwent craniotomy but did not receive a second set of VPC injections.

The stereotactically guided biopsy procedures and initial infusions of VPCs were well tolerated. None experienced hemorrhage, infection, seizure, or neurological deficit. At reoperation, gross-total resection was achieved in three cases, near-total resection in one case, and partial resection in the other. After craniotomy and the second set of injections of VPCs, one patient experienced a transient increase in a mild preoperative hemiparesis. Three patients remained at their preoperative Karnofsky level 6 weeks after treatment, and one patient improved from 80 to 90. The patient in Case 4 developed fever and purulent leakage from his wound on the 12th day postcraniotomy. Follow-up MR images demonstrated an abscess in the subgaleal, epidural, and subdural spaces and around the margin of the tumor resection. Craniotomy for debridement and administration of antibiotic medications failed to control the infection. The patient developed renal insufficiency and died 10 days later. Cultures obtained from all components of the abscess grew *Staphylococcus aureus*. Bacteriological cultures prepared from each vial of VPCs used in all five cases and the stereotactic infusion apparatus were consistently free of bacterial contamination.

During the 3 months of follow up, the four surviving patients showed no sign of toxicity from the treatment. None experienced hemorrhage, infection, seizure, or new neurological deficit. Patterns of marginal enhancement typical of a tumor resection cavity were observed on MR imaging (Fig. 2). On longer follow up, all patients had developed local recurrence of their disease (Table 2). One died at 6 months and one at 8 months posttreatment; the two others were still alive at 18 and 14 months posttreatment.

Histological and Immunohistochemical Findings

On macroscopic examination of the specimens obtained at resection we identified all of the infusion columns. Each had a cortical entry point, marked by a small amount of hemorrhage, and a discernible track. The width of the tracks varied from 0.2 to 0.4 cm, reflecting the different degrees of hemorrhage and tissue breakdown. No grossly
purulent debris was noted in association with any of the paths or within the overlying subarachnoid space. At least one needle track passed through grossly solid tumor in each case; other tracks traversed regions of infiltrated or marginal brain. With the exception of the needle trajectories, the macroscopic appearance of the specimens did not differ significantly from standard malignant glioma resection specimens.

Malignant high-grade glioma was demonstrated on light microscopic analysis in all cases, with histological evidence of frank recurrent glioblastoma in most specimens. Most specimens also exhibited histological features typical of treatment effects, such as radiation damage. The needle paths were characterized by the presence of fresh blood within the lumina as well as variable numbers of lipid-laden macrophages. The infusion cavities in some patients (Cases 3 and 5) contained numerous macrophages with relatively less fresh blood; in others (Cases 2 and 4) there was more fresh blood than collections of macrophages. Three patients (Cases 2, 4, and 5) had small acute infarcts adjacent to the needle tracks that were characterized by central thrombosed blood vessels, necrotic tissue, and polymorphonuclear cell infiltrates. No infectious organisms were noted in the specimens. Small focal infiltrates of small T-cell lymphocytes (Fig. 3) were noted in perivascular Virchow–Robin or overlying subarachnoid space, away from the needle tracks.

Discrete nodules of positively stained cells were demonstrated on TK-immunohistochemical studies in three of the patients (Cases 2, 4, and 5). Within the nodules the percentage of TK-immunopositive cells was less than 30% (Fig. 4). In Case 4, four such nodules were noted; in Cases 2 and 5, only one and two nodules were present, respectively. All of the nodules were within or adjacent to the needle tracks or in Virchow–Robin spaces less than 0.1 cm from a track.

Correlation with the hematoxylin and eosin–stained sections showed that these nodules were composed of loosely arranged elongated cells with vesicular nuclei and somewhat prominent nucleoli, in a slightly myxoid background. These cells were not actively proliferating, as determined by MIB-1 labeling; the only MIB-1–positive cells in these nodules were rare macrophages. The nuclei of the cells comprising these nodules were cytologically different from those of the surrounding tumor cells; the tumor nuclei were more irregular and hyperchromatic. In addition, the surrounding tumor cells and reactive astrocytes stained strongly immunohistochemically for GFAP, whereas the cells within the TK-positive nodules were GFAP-negative. No T-cell infiltrate was found immediately adjacent to the TK-positive nodules.

Quantification of TK Activity

The TK activity was measured in tissue obtained from Case 2. Kinase activity was measured in terms of phos-
phorylation of both thymidine and GCV. Two samples were studied: one, composed of solid tumor tissue, included the infusion column, and the other, composed of tumor-infiltrated brain, was 5 to 10 mm away from an infusion track. As anticipated, in both samples, phosphorylation of thymidine, which reflects the combined activity of the small amount of HSV-derived TK and the more prevalent mammalian TK, exceeded the phosphorylation of GCV, which reflects only the activity of HSV-derived TK (Table 3). Consistent with a greater density of both VPCs and tumor cells, the solid tumor tissue containing the infusion column had between two and three times the kinase activity as that of tumor-infiltrated brain distant from the column.

Although these measurements constitute proof of the feasibility of in vitro measurement of the enzymatic activity of a therapeutic gene, it is likely, given the immunohistochemical findings, that this activity originated from the VPCs rather than from transduced tumor cells. Even so, the activity measured is quite low relative to that of the positive control (a rat 9L gliosarcoma cell line stably transfected with tk had a GCV kinase activity of 31.1 nmol/mg protein/hour).

Discussion
In this study we confirm the feasibility of stereotactically guided infusion into malignant gliomas of cells that produce retroviral vectors. The combination of an initial limited exposure and a secondary more extensive treatment was well tolerated. There were no adverse consequences of the infusion procedure, implantation of a xenograft producer cell line, or release of retroviral vector in four of the five patients. The one severe complication encountered in this group, namely death from wound infection, intracranial abscess, and multisystem failure in a 79-year-old patient, raised the possibility of infection induced by the VPC injections. Cultures of VPCs remaining from the aliquots injected, of tubes used to dilute and transport the cells, of buffer solutions used to dilute the
Infiltrated brain 0.77 0.09 0.3

810

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Both before and, in a separate case, after infusion.12 The

activity of a therapeutic foreign gene in this paradigm.

showing that active enzyme is actually produced is crit-

tical to confirming the assumed mechanism of such pro-

drug-activating enzyme gene therapy paradigms.

Our studies showed modest T-cell infiltration in the vicinity of the infusion tracks in the absence of clinical

signs of cerebritis or meningitis. We remain uncertain as to the radiographic correlate of such inflammation but note that the thickness and intensity of marginal enhance-

ment of the resection bed did not differ between the injected and un.injected areas.

In this study we have demonstrated that the \(k\)-GCV paradigm, at the VPC levels, infusion parameters, and doses that we used, has no discernible clinical or patho-

logical effect on malignant recurrent gliomas. We identify two major barriers: 1) lack of dissemination of either VPCs or vector away from the infusion site; and 2) a very

low rate of tumor cell transduction. These factors do not pertain in tissue culture, in which the paradigm has been so effective.19 The fact that xenograft tumors in immuno-

competent animals regress may reflect the impressive im-

mune response that accompanies VPC injection.2,11,19 The

therapeutic efficacy seen in gliomas in nude mice may re-

fect efficient retroviral infection of tumor cells. This, in

turn, may result from the high mitotic rate of these tumors,

which is greater than that found in human gliomas.18

Clearly, improved methods of delivery and increased rates

of transduction will be required before this or similar strat-

egies are of clinical benefit.

More significantly, this study demonstrates both the

feasibility and the importance of incorporating within

gene therapy trials a quantitative assessment of the expres-

sion of a therapeutic gene and its effects on diseased tis-

sue. This experimental protocol, which includes an inter-

val between a limited infusion and tissue harvest before

more extensive treatment, provides a valuable opportuni-

ty to quantify gene expression and its consequences. Such

gene marking, accompanied by pathological, immunologi-

cal, and biochemical analyses of tissues harvested, is es-

sential to the proof of concept for novel gene therapies and

should provide a quantitative basis for choice of dose and

delivery parameters. Critical issues that warrant assess-

ment include 1) the tolerance of the delivery method; 2) the
cell type, frequency, and distribution of transduction of the therapeutic gene; 3) the amplitude of the intermediate
effect of the therapeutic gene; 4) the immune response to the treatment; 5) the strength of the therapeutic effect

achieved; and 6) the nature of any adverse consequences.

Assumptions regarding these issues should be tested in

limited proof of concept and Phase I trials by using tem-

plates such as that presented in this study before novel gene therapy techniques are disseminated for widespread

multinstitutional and commercial use.

Acknowledgments

The authors thank Dr. J. Hines (Johns Hopkins University) for measuring TK activity in the resection specimens, Dr. K. Bankiewicz (National Institutes of Health) for collaboration in developing the stereotactic infusion apparatus, Ms. Kristen Suling (Massachusetts General Hospital—Molecular Neuro-Oncology Laboratory) for valuable technical assistance, and Ms. Sarah Jhung (Molecular Neuro-Oncology Laboratory) for help with image analysis.

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G. R. Harsh, et al.


Manuscript received September 24, 1999. Accepted in final form January 16, 2000. This work was supported in part by a Program Project grant from the National Cancer Institute (5PO1CA69246) to Drs. Harsh, Chicocca, and Hochberg.

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