Assessment of Ganciclovir Toxicity to Experimental Intracranial Gliomas following Recombinant Adenoviral-mediated Transfer of the Herpes Simplex Virus Thymidine Kinase Gene by Magnetic Resonance Imaging and Proton Magnetic Resonance Spectroscopy

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ABSTRACT
Magnetic resonance imaging and in vivo localized 1H magnetic resonance spectroscopy were used to evaluate a gene therapy approach for treating experimental brain tumors. This approach involved the use of an adenoviral vector to transfer the herpes simplex virus thymidine kinase (HSVtk) gene into intracerebral 9L gliosarcomas in rats followed by systemic administration of the antitherapeutic agent ganciclovir. Magnetic resonance imaging quantitation of changes in intracranial 9L tumor doubling times revealed a significant variation in therapeutic response. Localized 1H magnetic resonance spectra of 9L tumors treated with Ad.RSVtk/ganciclovir revealed a dramatic increase in the resonance intensity at 0.9-1.3 ppm, corresponding to mobile lipids and/or lactate. Changes in intracranial tumor doubling times correlated with changes in 1H tumor magnetic resonance spectra, suggesting that specific changes in tumor metabolite levels may be predictive of the effectiveness of this gene therapy approach.

INTRODUCTION
Malignant tumors (glioblastoma multiforme) of the CNS constitute a major therapeutic problem because of their frequency of occurrence and extremely poor prognosis (1, 2). Because current therapeutic interventions have failed to significantly improve the survival rate of patients with malignant tumors of the CNS, novel treatments are being evaluated. Among these, gene therapy holds great promise for neuro-oncology. The basic goal of gene therapy is to transfer a gene into tumor cells, allowing sensitization of the cells to drugs that are nontoxic until metabolized by the gene product (3). These so-called “suicide genes” are designed to produce an exploitable biochemical difference between normal and tumor tissues in order to create a selective therapeutic advantage (3, 4). Selectivity may be potentially achieved through the use of vectors and promoters which limit the insertion and expression, respectively, of the gene to tumor cells. A review of suicide genes that have been characterized to date has recently been published by Moolten (5).

One suicide gene that has undergone extensive evaluation for the treatment of brain tumors is the HSVtk gene (6–13). Cells which express HSVtk phosphorylate antitherpetic agents, such as GCV, an analogue of guanosine, to its monophosphate form. Following further phosphorylation by cellular kinases to its triphosphate form, this analogue can then be utilized for incorporation into DNA. The presence of this analogue in DNA has been reported to inhibit DNA polymerase, causing toxicity (14).

In this study, we have used a recombinant adenoviral vector (Ad.RSVtk) to transfer the HSVtk gene to intracranial 9L tumors in the rat followed by systemic administration of GCV. Animal survival studies revealed that this approach produced a significant therapeutic effect over control animals. MRI has been recently reported as a noninvasive method for quantitating the tumor doubling times of individual intracerebral rat brain tumors and changes in growth rates resulting from therapeutic intervention (15). In this study, intracranial tumor doubling times were quantitated in individual animals by measuring the tumor volume at successive time intervals from MR images. MRI quantitation of tumor doubling times for individual rats revealed a significant variation in response to Ad.RSVtk/GCV. Localized 1H MR spectra of 9L tumors treated with Ad.RSVtk/GCV revealed a dramatic increase in the resonance intensity at 0.9-1.3 ppm, corresponding to mobile lipids and/or lactate. Correlation of the changes in intracranial tumor doubling times with changes in metabolite levels of Ad.RSVtk/GCV-treated tumors could provide a noninvasive method for evaluating the effectiveness of gene therapy.
9L tumors revealed that in vivo $^1$H MRS may be useful for predicting therapeutic efficacy of this gene transfer approach.

MATERIALS AND METHODS

Construction of Ad.RSVtk. Construction of Ad.RSVtk was described elsewhere (12). In brief, sequences containing the tk gene were subcloned into the adenoviral shuttle plasmid pAdRSV4. pAdRSV4 contains a small multiple cloning site flanked by the Rous sarcoma virus promoter element on the 5’ end and a polyadenylation signal on the 3’ end. This adenoviral vector was generated by homologous recombination into the Ad5 (sub360) backbone, and plaques were isolated and amplified as described (12). Appropriate recombinant plaques were subjected to one additional round of plaque purification, amplified and purified for use. Titters for each preparation were determined by limiting dilution on 293 kidney cells.

Cell Culture Conditions and Induction of Gliomas. 9L gliosarcoma cells were grown as monolayers in 75-cm² sterile plastic flasks in modified Eagle’s MEM with 10% FCS and antibiotics. Cells were grown to confluence at 37°C in an atmosphere containing 95% air and 5% CO₂, harvested, counted, and resuspended in MEM without serum for inoculation. Intracerebral brain tumors were induced in anesthetized (ketamine, 87 mg/kg, + xylazine, 13 mg/kg body weight i.p.) male Fischer 344 rats weighing between 125 and 150 g. A small skin incision over the right hemisphere was made. A high-speed drill was used to create a 1-mm diameter burr hole through the skull. Stereotactic inoculation of $10^9$ 9L tumor cells in 5 µl serum-free culture medium in the right forebrain at a depth of 3 mm was accomplished. The burr hole was filled with bone wax to minimize extracerebral extension of the tumor tissue. The skin was sutured, and the rats were allowed to recover.

Survival Study. To determine the effects of Ad.RSVtk/GCV treatment on the survival of normal rats and rats with intracerebral 9L tumors, three cohorts of animals which consisted of eight animals per cohort were used for the following studies. One cohort was inoculated intracerebrally with $20 \mu l$ Ad.RSVtk ($1.2 \times 10^{11}$ pfu/ml) followed by administration of GCV (15 mg/kg 2 times daily, i.p.) beginning 24 h later. The other two cohorts were inoculated intracerebrally with $5 \times 10^9$ 9L tumor cells. At 7 days post 9L cell implantation, one cohort received a $20 \mu l$ Ad.RSVtk injection and the other $20 \mu l$ of Ad.RSVlacZ ($1.6 \times 10^{11}$ pfu/ml). Each of these latter two cohorts were treated with GCV beginning 24 h following viral inoculation for a 14-day period. Following adenoviral administration to each group of animals, 40 µg dexmethasone was given (i.p.) in 0.5 ml saline to minimize the potential of cerebral edema. At 24 h following injection, rats were given 4 µg dexmethasone/ml drinking water.

MR Imaging and $^1$H Spectroscopy. A separate group of six rats with intracerebral 9L tumors was used for evaluation of Ad.RSVtk/GCV treatment with MRI. Of these six animals, three were also evaluated by $^1$H MRS. One animal treated with Ad.RSVlacZ/GCV was also evaluated using MRI. All in vivo MR experiments were performed on a Spectroscopy Imaging Systems Corporation System equipped with a 7.0 tesla, 18.3-cm horizontal bore magnet (300-MHz proton frequency). For MRI examination, rats were anesthetized with a ketamine/xylazine mixture and maintained at 37°C inside the magnet using a heated circulating water blanket. MRI of rat brains was initiated between 8 and 10 days following cell implantation and repeated every other day. The rat head was positioned inside a 4-cm diameter birdcage RF coil, which was designed and constructed with ear bars to secure the position of the head during the acquisition. For each MRI session, a single-slice gradient-recalled echo image was acquired with 1-mm “saturation cross-hairs” imprinted on the axial and coronal images to facilitate rapid and reproducible positioning of the animal as described previously (15). Multislice axial images were acquired using a standard spin echo sequence. T2-weighted images through the rat brain were produced using the following parameters: TR/TE = 3500/60, FOV = 30 × 30 mm using a 128 × 128 matrix, slice thickness = 0.5 mm, and slice separation = 0.8 mm. Twenty-five MR images were obtained following acquisition of a second set of 25 slices with a slice offset of 0.4 mm. This provides a contiguous image data set of the rat brain as described previously (15). For tumor volume measurements at each time point, the area (volume) of tumor visualized in each slice as a hyperintense region was manually outlined using a region of interest program provided with the Spectroscopy Imaging System Corporation System MR software. The outlined tumor area in each cross-sectional image was multiplied by the slice separation (0.4 mm) to calculate the tumor volume in each slice. Tumor volumes of individual slices were summed to yield the total volume of the tumor as described previously (15). After pretreatment, $T_d$ for each glioma was determined from four image data sets; rats underwent the gene transfer procedure as described below. Collection of images was continued every other day until appearance of a significant neurological deficit.

Water-suppressed spatially localized $^1$H spectra were acquired on three rats 4–6 days following Ad.RSVtk/GCV treatment using a previously described method (16). In brief, spatial localization utilized a hybrid approach by combining two-dimensional ISIS (17) and spectroscopic imaging (18) to define contiguous slices along the axis of the ISIS column. The adiabatic spin-echo pulse, solvent suppressive adiabatic pulse (19), was incorporated into the ISIS-spectroscopic imaging sequence. The position of the ISIS column was determined from surface coil MR images (20). A 16-mm diameter surface coil was used for obtaining the surface coil MR images and for the $^1$H MRS studies. Localized in vivo $^1$H spectra were acquired from 25-µl tissue volumes. All tumors underwent histopathological examination for cellular changes.

Determination of Tumor $T_d$. Tumor volumes measured using MRI in individual animals were plotted against hours after cell implantation to determine the growth rate of the intracranial 9L gliomas (15). By using the equation for exponential growth, $V(t) = V_0 \times 10^k$, where $V(t)$ is tumor volume at time $t$, $V_0$ is the initial tumor volume, and $k$ is the tumor growth rate constant, the volumetric $T_d$ was calculated from the expression, $T_d = \log 2/k$. The tumor growth rate constant or $T_d$ was obtained from an exponential curve fit of the volume data points using a least-squares algorithm. This analysis was used to determine both the pretreatment and posttreatment intracerebral $T_d$ for each animal ($n = 6$). We estimated posttreatment $T_d$ using the above-mentioned monoexponential growth model for those
treated data points which followed a linear curve on a semi-log plot before regrowth of the tumor had occurred.

MR Gene Therapy Protocol. Following determination of pretreatment intracerebral 9L tumor Td from four MRI scans, a stereotactic injection of recombinant adenovirus (Ad.RSVtk or Ad.RSVlacZ) was administered through the central axis of the tumor through the same burr hole used for tumor cell implantation. The needle was lowered to the base of the tumor mass and at 2-mm distances during retraction of the needle through the center axis of the tumor. Administration of recombinant adenovirus to the 9L tumor was done at approximately 16 days after tumor implantation, and 40 μg dexamethasone was administered (i.p.) in 0.5 ml saline to minimize the potential of cerebral edema. All rats were given 4 μg dexamethasone/ml drinking water 24 h following recombinant adenoviral treatment. At 24 h following injection of Ad.RSVtk, treatment with GCV (15 mg/kg i.p., twice daily) was initiated. Control rats were treated similarly except that a control virus was used which contained lacZ (Ad.RSVlacZ).

RESULTS
Animal survival studies revealed that administration of Ad.RSVtk/GCV to intracerebral 9L tumors resulted in improved animal survival. Analysis of the survival data using Kaplan-Meier survival analysis was accomplished (21). Shown in Fig. 1 is the Kaplan-Meier survival curves for the group of rats treated with Ad.RSVtk/GCV and Ad.RSVlacZ/GCV. Log rank statistical analysis used to compare the survival distributions of rats treated with Ad.RSVtk/GCV and Ad.RSVlacZ/GCV showed a significant difference in survival between the two groups (P = 0.0006). Survival of rats without tumors was unaffected by Ad.RSVtk/GCV treatment over the 40-day study.

A growth curve for an intracranial 9L glioma treated with Ad.RSVlacZ/GCV is shown in Fig. 2. The tumor volumes obtained before and after Ad.RSVlacZ/GCV administration are denoted by (□) and (●), respectively. The pretreatment volumetric data points were used for the exponential curve fit and revealed that administration of the virus containing a control gene (lacZ), in combination with systemic administration of GCV, did not affect the exponential growth rate of the intracerebral 9L tumor.

MRI examination of rats treated with Ad.RSVtk/GCV revealed significant reductions in tumor Td as shown in Fig. 3. The pretreatment intracerebral 9L tumor volume measurements are shown (□) along with the least-squares fit. As shown in Fig. 3, a marked retardation of 9L tumor growth was observed following Ad.RSVtk/GCV administration [Fig. 3, data points (●)] in this study. 9L tumors were infected with Ad.RSVtk following acquisition of the fourth volumetric data point. A 24-h time period was allowed to pass in order for the gene product, HSVtk, to be expressed within the tumor cells. GCV administration was then initiated and maintained throughout the duration of the study. Pretreatment and posttreatment 9L tumor Ts were determined from volumetric data points before regrowth occurred. A statistically significant difference (P < 0.05) between the mean pretreatment and posttreatment intracranial tumor Ts was observed by MRI volumetric studies; however, a large variation in antitumor activity between individual animals was observed as shown in Fig. 3 and summarized in Table 1. Rats A, B, E, and F died following the last plotted MRI-determined volumetric time point.

Shown in Fig. 4 are in vivo localized 1H MR spectra of rat brains with untreated and Ad.RSVtk/GCV-treated 9L tumors, respectively. In Fig. 4, a series of spectra from 25-μl voxels along a column through the cerebral hemispheres of the rat are...
displayed. Spectra of untreated gliomas revealed greatly reduced $N$-acetyl aspartate levels and reduced levels of creatine as compared to the contralateral hemisphere. The lipid/lactate resonance from untreated gliomas and healthy brain was hardly visible. In a rat which received GCV treatments for 4 days following Ad.RSVtk administration, tumor $^1$H spectra revealed a dramatic increase in the lipid/lactate resonance intensity (Fig. 4B). The spectra shown in Fig. 4B corresponds to those in Fig. 3A and Rat A in Table 1. Similar changes in the $^1$H spectra of 9L tumors were observed for all three rats treated with Ad.RSVtk/GCV. Shown in Fig. 5 is a hematoxylin and eosin-stained histological coronal section of the brain corresponding to the same rat from which the treated $^1$H MR spectra are shown (Fig. 4B). Histological examination revealed a large area of
central necrosis (Fig. 5, arrows) within the treated 9L tumor mass, which was not typically observed in untreated 9L tumors.

**DISCUSSION**

The survival rate of patients with primary malignant tumors of the CNS (glioblastoma multiforme) has not improved appreciably with current treatment strategies (1, 2). Because of the lack of progress, alternative therapeutic approaches are being evaluated such as the transfer of genes encoding oncolytic molecules using viral or synthetic transducing agents (for review, see Ref. 5). In this study, we have used MR techniques to evaluate the therapeutic effectiveness of a recombinant replication defective adenoviral vector as a vehicle for transferring the HSV1k gene into 9L tumors in vivo. The data reported here confirm that Ad.RSV1k confers GCV toxicity to intracerebral 9L gliosarcomas and that MRI and MRS offer excellent methods for noninvasively monitoring the progress of the treatment in individual animals.

The therapeutic response of orthotopic brain tumors has traditionally been quantitated using animal survival, tumor weights following excision, or colony-forming assays of cells cultured from the in situ tumor as biological end points (22–32). Results from the animal survival study reported here revealed a significant improvement in survival of animals with intracerebral 9L tumors following treatment with Ad.RSV1k/GCV. Variation in the 9L therapeutic response was directly observed in this present study using MRI as demonstrated in Fig. 3. Furthermore, a significant variation in pretreatment tumor $T_d$ was observed here as previously reported for the 9L tumor using MRI (15).

MRI allows quantitation of intracranial tumor volumes to be noninvasively and, thus, repeatedly monitored over the life span of individual animals. However, the clinical evaluation of treatment efficacy in CNS cancers is more complex (33–36). This is due in part to the fact that improvement in patient function is multifactorial and that changes in neurological deficits may be unrelated to changes in tumor size. Furthermore, the use of computed tomography and MRI scans in the clinical setting do not permit absolute quantitation of tumor volume. Because of the difficulty in measuring the size of intracranial tumors in humans, brain tumor therapeutic trials are usually reported as median survival time and median time to progression as a quantitative measure of response (33–36). This measurement of response is used despite the fact that these parameters may not correlate with therapeutic efficacy in an individual patient. Therefore, methods which would allow for accurate quantitation of the therapeutic response in individual patients would be valuable. In this regard, the potential of MRS for

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**Fig. 4** In vivo localized $^1$H spectra of a rat brain with a 9L gliosarcoma in the right hemisphere. A, rat with an untreated 9L tumor and B, rat with a 9L tumor that had been treated with GCV for 4 days following stereotactic administration of Ad.RSV1k. Note the increased resonance at 1.3 ppm in the treated 9L tumor. Each spectrum arises from 25-$\mu$L voxels along a two-dimensional ISIS column through the rat brain. Spectral assignments are mobile lipids and/or lactic acid (0.9–1.3 ppm), N-acetyl aspartate (2.0 ppm), glutamate/glutamine (2.1–2.5 ppm), creatine and phosphocreatine (3.0 ppm), and choline groups (3.2 ppm).

**Table 1** Summary of MRI results in which a replicative defective adenovirus containing the herpes thymidine kinase gene (Ad.RSV1k) was injected stereotactically into intracerebral rat 9L gliomas followed by systemic GCV administration

<table>
<thead>
<tr>
<th>Rat</th>
<th>$T_d$ Pre-ganciclovir (h)</th>
<th>$T_d$ Post-ganciclovir (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>43</td>
<td>213</td>
</tr>
<tr>
<td>B</td>
<td>44</td>
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<td>C</td>
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<td>276</td>
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<tr>
<td>D</td>
<td>51</td>
<td>206</td>
</tr>
<tr>
<td>E</td>
<td>59</td>
<td>N/A</td>
</tr>
<tr>
<td>F</td>
<td>37</td>
<td>487</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>49 ± 4*</td>
<td>429 ± 143*</td>
</tr>
</tbody>
</table>

* Note: Rats A, B, E, and F died following MR examination due to anesthesia. Rats C and D were euthanized following appearance of neurological deficits due to tumor burden.
monitoring therapy of brain tumor patients was investigated to determine whether specific changes in tumor metabolite levels could be correlated with therapeutic effectiveness.

*In vivo* $^1$H MR spectra of 9L tumors treated with Ad.RSVtk/GCV revealed a large increase in the peak at 0.9–1.3 ppm, which corresponds to mobile lipids and/or lactic acid. An *ex vivo* high-field MR spectroscopy study of biopsies obtained from high-grade astrocytomas has recently reported that MR-visible lipids were observed in necrotic foci (37). In fact, the intensity of the lipid signals at 2.0, 1.3, and 0.9 ppm increased with increasing amounts of necrosis within the tissue sample (37). The increased $^1$H spectral signal intensity of the treated *in vivo* 9L tumors in this present study would be consistent with increased tumor necrosis due to GCV-induced cell death. Preliminary histological analysis of 9L tumors treated with Ad.RSVtk/GCV (Fig. 5) revealed a large area of central necrosis within the tumor mass, which is consistent with the observations of Kuesel et al. (37). The resonance at 0.9–1.3 ppm could also contain a contribution from lactic acid. This could arise as a consequence of cell death or due to tumor ischemia. A recent report showed that endothelial cells of tumor blood vessels could be transduced with the HSVtk gene using a retrovirally mediated gene transfer (38). This study suggested that a reduction in tumor blood supply could be an important aspect of HSVtk transduction-mediated tumor regression (38). Clearly, further $^1$H MRS studies incorporating spectral editing techniques are necessary to further characterize the relative contributions of lipid and lactate to the Ad.RSVtk/GCV-treated 9L tumor spectra.

The use of MRS in the clinical setting will depend on its capability for diagnosis, prognosis, or individualization of treatment. These capabilities may be advanced through the careful correlation of specific therapeutic-induced changes in MRS-observable metabolite levels with MRI determination of therapeutic effectiveness using rodent brain tumor models. Evaluation of new treatment strategies using rodent brain tumor models with MRI/S offers many advantages, including improving characterization of dose-response relationships, drug uptake and retention studies, mechanism of action studies, and correlation of MRS-observable metabolic changes with cell kill for use in clinical evaluation of treatment. This type of correlation can provide insights into specific metabolic change(s) which are indicative of therapeutic efficacy.

Gene transfer techniques are increasingly being developed and explored for the treatment of malignant primary tumors of the brain such as glioblastoma. Because the efficiency of the gene transfer procedure can vary for many reasons (including type of vector used, infection technique, stability of the particular gene and expressed protein, viral titer of injected solution, inaccurate coordinates used for stereotactic injection, etc.), it would be especially valuable to have a method for noninvasive assessment of treatment effectiveness. The use of MRI/S in future gene therapy experimental protocols should greatly facilitate our understanding of how best to optimize each new antitumor therapy.

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Assessment of ganciclovir toxicity to experimental intracranial gliomas following recombinant adenoviral-mediated transfer of the herpes simplex virus thymidine kinase gene by magnetic resonance imaging and proton magnetic resonance spectroscopy.

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