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In Vivo ^{31}P MRS Evaluation of Ganciclovir Toxicity in C6 Gliomas Stably Expressing the Herpes Simplex Thymidine Kinase Gene

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Phosphorus MRS was evaluated as a monitor of tumour therapeutic response to the herpes simplex virus thymidine kinase suicide gene therapy paradigm. *In vivo* ^{31}P spectra were obtained from subcutaneous rat C6 gliomas constitutively expressing the HSVtk gene post treatment with ganciclovir (GCV, 15 mg/kg i.p., twice-daily). Significant regression ($p<0.1$) of tumour volume was observed 10 days after beginning GCV administration. However, no changes in tumour pH or energy metabolites from pre-treatment values were observed. High-resolution ^{31}P spectra of tumour extracts revealed a statistically significant reduction in the phosphocholine to phosphoethanolamine ratio six days post-GCV administration. These results indicate that the HSVtk/GCV-induced killing of tumours is not associated with corresponding changes in ^{31}P MRS-observable energy metabolites and pH. The observed reduction in the PE/PC ratio may provide a non-invasive *in vivo* indicator of therapeutic efficacy. © 1996 by John Wiley & Sons, Ltd.

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INTRODUCTION

The limited differential susceptibility of normal and neoplastic cells to tumour therapies has resulted in the development of alternative treatment strategies. One such strategy involves the introduction of non-mammalian genes into tumour cells in order to confer artificial differences between normal and malignant cells. During the past decade, the potential of the gene therapy approach to significantly enhance the outcome of neuro-oncology treatment has been clearly demonstrated in animal models.[1] Numerous workers have successfully treated experimental central nervous system tumours by introducing the herpes simplex virus type 1 thymidine kinase (HSVtk) 'suicide gene' via viral transduction of the tumour cells.[2–11] The cells which express HSVtk phosphorylate anti-herpetic guanine nucleoside analogs, such as ganciclovir (GCV), that are poor substrates for the endogenous mammalian thymidine kinase. Subsequent phosphorylation by endogenous kinases results in the formation of the triphosphorylated form of GCV, which is incorporated into DNA and terminates DNA synthesis.[12,13] Thus, systemic toxicity is minimized by restricting the generation of cytotoxic metabolites of the drug to within the transduced cells.

The HSVtk/GCV gene therapy approach has proceeded to clinical trials at multiple centres.[14] However, transduction efficiency and the extent of HSVtk expression is highly variable, making the clinical application of the protocol complex. Currently, the response of human intracranial tumours to therapy is quantitated as median survival time or median time to tumour progression.[15–17] These variables, however, do not correlate well with the therapeutic response in an individual patient. Furthermore, these are relatively late manifestations of changes in the tumour, precluding their use in tailoring the treatment to the individual. Non-invasive detection of early metabolic and proliferative response of a tumour in an individual patient would be valuable in the clinical application of this gene therapy strategy. Such a tool could guide re-transduction of the tumour and allow modulation of GCV dosages to maximize the therapeutic effect.

In vivo ^{31}P MRS has been used extensively as a non-invasive assay of treatment–response in human and animal brain tumours to a wide variety of therapeutic interventions.[18–26] This study examines the value of ^{31}P MRS as an early monitor of tumour response to the HSVtk/GCV gene therapy paradigm. Following GCV administration, *in vivo* and *ex vivo* spectra were obtained from subcutaneous rat tumours derived from a recombinant C6 rat glioma cell line stably expressing the HSVtk gene. Changes in phosphorus metabolite ratios and tumour pH were correlated with changes in tumour volume and compared with pre-treatment values.

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MATERIALS AND METHODS

Induction, measurement and treatment of subcutaneous glioma

C6 rat glioma cells stably expressing the herpes simplex virus thymidine kinase gene (C6BSTK) were grown in culture in the presence of 1 mg/ml neomycin as previously described.[6] Cells (5×10^5) were implanted subcutaneously in the right flank of 15 2-week old athymic nude rats in 0.1 ml of serum-free medium. Tumour volume was quantified from caliper measurements every 2 days beginning on the 13th day post-implantation using the following equation: volume=0.5 (length × width^2).[27] GCV (15 mg/kg) was administered i.p. twice daily beginning 31 days post-tumour implantation when tumour volume reached approximately 1 cm^3.

In vivo ^{31}P MRS

Rats were anaesthetized by an i.p. injection of a 90/13 mg/kg of a ketamine/xylazine cocktail. ^{31}P spectra (121.4 MHz) of C6BSTK subcutaneous tumours (n=4) were obtained using a Varian system equipped with a 183 mm horizontal bore 7 T magnet and a 0.8 cm diameter surface coil. Spectra were acquired using the following parameters: 4K data points, spectral width of 8000 Hz, repetition time of 2.3 s, 500–1000 signal averages, and a pulse width of 8 μs. Spectra were apodized with a 40 Hz line-broadening function prior to Fourier transformation. Resonance areas were quantified using a spectral deconvolution software routine (Varian Associates Inc.). Intracellular tumour pH was calculated from the difference in PCr and P_i chemical shift as previously reported.[28] Statistical analysis of metabolite ratios of pre- and post-treatment tumours was carried out using the two-tailed Student's t-test.

Ex vivo ^{31}P MRS

Subcutaneous C6BSTK tumours approximately 1 cm in diameter were assigned to either a non-treated control group (n=4) or a group treated for 6 days with GCV (n=7). This treatment protocol resulted in tumours with volumes of approximately 1 cm, allowing direct comparison to pre-treatment controls. Subcutaneous gliomas were resected and immediately extracted in 0.6 M perchloric acid at 4°C. Extracts were neutralized with 0.6 M potassium bicarbonate, centrifuged to remove all insoluble potassium perchlorate, lyophilized, and then resuspended in phosphate-free buffer (pH 7.2) containing 30% D_2O and methylene diphosphonic acid (MDA). Phosphorus spectra were acquired at 161.9 MHz on a Bruker AMX400 system using the following parameters: 8K data points, spectral width of 8000 Hz, 10 s repetition time, 10,000 signal averages, and a 90° flip angle. The deuterium signal served as a magnetic field lock, and WALTZ-16 decoupling was used. Chemical shifts were referenced to MDA and peak areas determined by spectral deconvolution. The ratio of the phosphocholine (PC) to phosphoethanolamine (PE) peak areas was calculated and the values of the two tumour extract groups were statistically compared by the two-tailed Student's t-test analysis of the means.

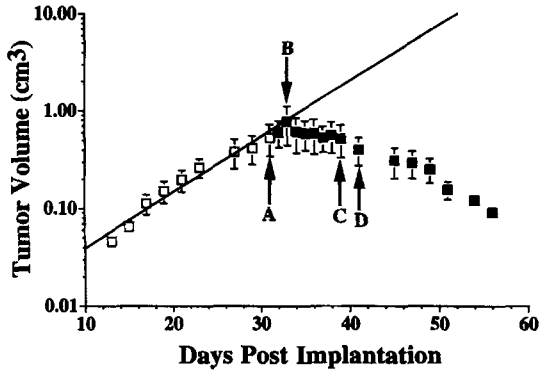

Figure 1. The effect of GCV on subcutaneous C6BSTK tumour growth. Tumour volumes were measured before (□) and after (■) twice-daily administration of 15 mg/kg GCV. Arrows indicate days in which *in vivo* ^{31}P-NMR spectra were obtained. (A) Day 0 spectrum acquired immediately prior to the first GCV dose, (B) after 2, (C) after 8, and (D) after 12 days of GCV treatment, n=5±SE.

RESULTS

Subcutaneous C6BSTK tumours were measurable about 12 days after induction and reached a volume of approximately 0.5 cm^3 in 31 days. As shown in Fig. 1, subcutaneous tumours grew exponentially prior to GCV treatment. Tumour growth arrest was observed 2 days after initiation of twice daily treatents of GCV. Significant debulking (p<0.1) followed after 10 days of therapy. GCV administration was continued for a period of 25 days at which time the average volumes of the tumours decreased to approximately 0.06 cm^3.

In vivo ^{31}P MR spectra were acquired immediately prior to the first dose of GCV and on the 2nd, 8th and 12th days of GCV treatment corresponding to the arrows denoted as A, B, C and D, respectively, depicted in Fig. 1. In general, pH and metabolite levels of subcutaneous tumours are known to change as the tumours outgrow their blood supply.[29] To control for these changes, spectra from treated tumours were compared to spectra from untreated tumours of similar size. As shown in Fig. 1, there were no statistically significant differences between tumour volumes immediately prior to treatment and volumes after 8 days of GCV administration. Although statistically significant (p<0.1), the volumes of the tumours at day 12 were only minimally smaller. Thus pre-treatment spectra were suitable controls for comparison to spectra obtained over the initial 12 days of therapy. Representative *in vivo* spectra for untreated and GCV-treated tumours are shown in Fig. 2(A)–(D). No apparent changes in tumour pH or energy metabolites were observed despite the marked growth arrest and tumour regression observed (Fig. 1). A comparison of P_i/β-NTP, PCr/β-NTP and PME/β-NTP ratios and tumour pH over the course of GCV administration revealed no statistically significant change from the pre-treatment values as shown in Table 1.

MR observable phospholipid metabolites have been assessed as indicators of cancer cell function or tumour response to therapy.[30] As shown in Fig. 2, PME levels in C6BSTK tumours did not change upon GCV treatment. However, the PME peak consists of contributions from both phosphocholine (PC) and phosphoethanolamine (PE) and quantitation of these metabolites was not possible *in vivo* due to insufficient spectral resolution. We therefore performed ^{31}P MRS on tissue extracts in order to determine

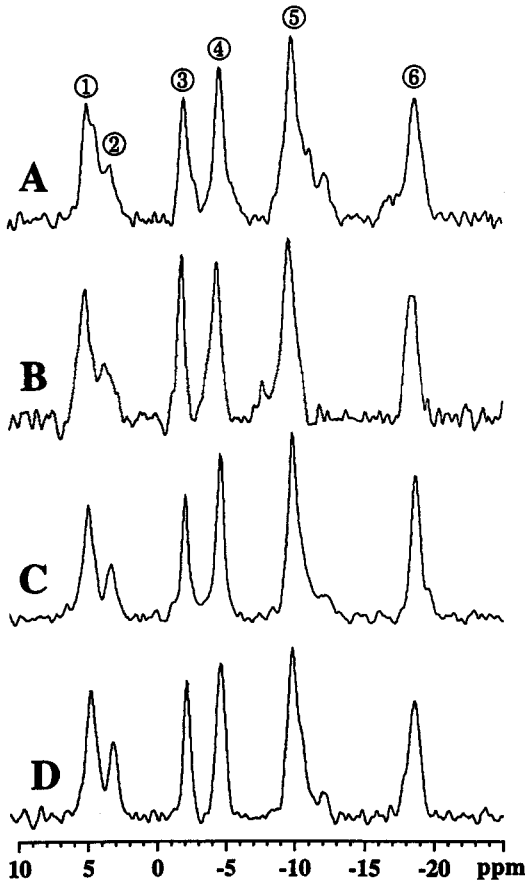

Figure 2. *In vivo* ^{31}P-NMR spectra of a subcutaneous C6BSTK glioma from a single rat obtained (A) before GCV administration, and after (B) 2, (C) 8, and (D) 12 days of GCV treatment, which correspond to the volumes denoted by arrows in Fig. 1. Resonance assignments are as follows: 1—phosphomonoester (PME); 2—inorganic phosphate (P_i); 3—phosphocreatine (PCr); 4—β-NDP and γ-NTP; 5—α-NTP, α-NDP and NAD^+/NADH; 6—β-NTP.

whether GCV-induced changes in phospholipid metabolites occur. Representative ^{31}P MR spectra of extracts of untreated and GCV-treated subcutaneous C6BSTK gliomas are shown in Fig. 3. The PC and PE resonances were well resolved in the extract spectra, revealing a PE/PC ratio of 4.67 ± 0.06 ($n=7$) in the untreated controls and 3.53 ± 0.11 ($n=4$) in tumours following 6 days of GCV treatment. The difference in PE/PC ratios between the two groups was statistically significant ($p<0.025$). The decrease in the PE/PC ratio occurred during the period of tumour growth arrest,

Table 1. *In vivo* metabolite ratios and pH of rat subcutaneous C6BSTK gliomas before and during the course of GCV treatment

Days post-treatment	P_i/β-NTP	PCr/β-NTP	PME/β-NTP	pH
0	0.29±0.09	0.63±0.06	1.06±0.21	7.29±0.02
2	0.28±0.10	0.59±0.17	1.29±0.10	7.36±0.06
	(p=0.77)	(p=0.82)	(p=0.87)	(p=0.23)
8	0.47±0.10	1.13±0.29	1.06±0.14	7.26±0.03
	(p=0.15)	(p=0.31)	(p=0.69)	(p=0.13)
12	0.32±0.03	0.67±0.10	0.82±0.12	7.26±0.04
	(p=0.29)	(p=0.18)	(p=0.81)	(p=0.45)

Metabolite ratios were calculated for individual spectra during GCV treatment (15 mg/kg, twice-daily). Values are means±SE (n=4). There are no statistically significant differences in the mean ratios from various days post-treatment compared to pre-treatment, as determined by a two-tailed Student's t-test.

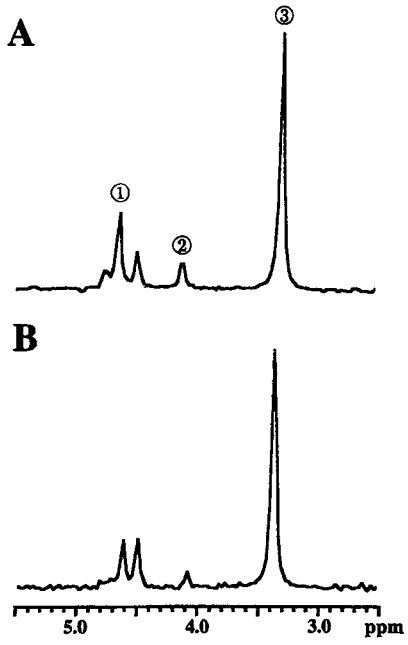

Figure 3. Representative high-resolution ^{31}P-MRS of perchloric acid extracts of 1 cm^3 subcutaneous C6BSTK tumours. (A) Control spectrum of untreated tumour, (B) spectrum obtained after 6 days of i.p. twice-daily administration of 15 mg/kg GCV. Resonance assignments: 1—phosphoethanolamine (PE); 2—phosphocholine (PC); 3—inorganic phosphate (P_i).

but before significant debulking of the tumour had occurred.

DISCUSSION

The HSVtk/GCV gene therapy system has been widely reported to be efficacious for the treatment of numerous tumour cell types both *in vitro* and *in vivo*.[3–11] In this study we have sought to determine whether MRS could be utilized as an early predictor of the efficacy of the HSVtk gene therapy paradigm. This was accomplished by examining a clonal cell ine (C6BSTK) which uniformly expresses the HSVtk gene, since it was anticipated that this approach would produce the maximal metabolic change induced by the HSVtk/GCV treatment paradigm. *In vivo* ^{31}P MRS spectra revealed no significant changes in energy metabolites or pH following GCV administration in spite of tumour growth inhibition. Furthermore, preliminary high resolution ^{31}P MRS studies of extracts obtained from cultured C6BSTK spheroids treated with GCV for up to 2 days, which resulted in reduced cell survival to approximately 10%, revealed the same lack of changes in ^{31}P metabolites as was found *in vivo* (data not shown).

This observation falls outside the two qualitatively distinct patterns of phosphorus metabolite changes associated with tumour treatment as described by Steen.[23] One response, exemplified by ischemia-inducing treatments, is characterized by a disruption of cellular energetics with decreased ATP and increased P_i when compared with age-matched untreated tumours or pretreatment tumour metabolite levels. The other response, commonly observed with chemotherapy and radiotherapy, is a paradoxical enhancement in the energy state of the tumour. The inability to elucidate a universal response to treatment requires that the efficacy of ^{31}P MRS as a therapeutic monitor be evaluated for each tumour type and treatment modality.

Moreover, the specific changes (or lack thereof) in metabolite levels observed by MRS may provide insights into the biochemical mechanisms of toxicity of an individual treatment method.

One explanation for our results pertains to the observation that C6 glioma[31] and other cells[32–34] treated with the HSVtk/GCV paradigm die by apoptosis. Apoptosis is an active process of 'cell suicide' in which high levels of NTP are sustained.[35] Thus, it is unlikely that there would be enough cells in ultimate stages of apoptosis, when high-energy metabolites are depleted, to impact the ^{31}P spectra. Another possible explanation for the lack of changes in phosphorus energy metabolites is the fact that triphosphorylated GCV is cytotoxic by interference with DNA replication rather than a direct metabolic effect on energy metabolism. Tozer and Griffiths argue that ^{31}P spectra reflect the oxygen supply of viable cells and that metabolic changes are induced when, for example, hypoxia ensues.[36] These results suggest that the gene therapy protocol examined in this study does not affect the overall tumour oxygenation state. Moreover, treatment-induced tumour growth retardation and debulking may actually improve the perfusion of the residual viable tumour cells and facilitate the removal of the P_i produced by dying cells. This was also not observed probably due to the small size of the C6BSTK tumours treated in this study.

The use of a cell line constitutively expressing the HSVtk gene enabled the effects of this therapeutic approach on tumour cell metabolism to be delineated from treatment-induced alterations in tumour physiology. However, *in situ* transduction of tumours with viral vectors, as is necessitated in clinical studies, introduces the suicide genes into the proliferating tumour cells themselves as well as the endothelial cells of the tumour neovasculature.[7,8] Ram *et al.* have demonstrated significant early destruction of tumour blood vessels in wild-type rat gliomas treated with HSVtk-retrovirus producer cells followed by GCV.[37] In contrast, they found that these vascular changes were not observed in solid gliomas derived from tumour cells which were pre-transduced with HSVtk before animal implantation. Therefore, *in vivo* transduction may impair tumour perfusion, creating tumour ischemia potentially observable by ^{31}P MRS. If changes in energy metabolites occur in tumours transduced *in vivo*, then it may be possible to use MRS for monitoring therapeutic responsiveness of individual tumours. Moreover, the results of this study suggest that such changes could be attributed to treatment-induced changes in tumour oxygenation, thereby allowing comparison of the cytotoxicity of this therapy to tumour cells versus endothelial cells.

The significant decrease in the PE/PC ratio observed in tumour extracts after 6 days of therapy offers a potential marker of early response to treatment. Although these changes were observed in tumour extracts, localized *in vivo* spectra obtained at lower field strengths and with ^{1}H decoupling may provide sufficient resolution to monitor these changes clinically in individual patients. This result also raises intriguing questions about the biological response of these tumours to this gene therapy strategy. There are two alternative interpretations of the observed decrease in the PE/PC ratio with therapy. First, the decreased ratio may be attributed to an increase in the proliferative fraction of the cells surviving the initial stages of treatment. This is consistent with results obtained with other tumour cell lines in spheroids and bioreactors that show a decreased PE/PC ratio with increased cellular proliferation.[38,39] Other tumour extract studies have shown an inverse correlation between PE/PC ratios and tumour growth rate in subcutaneous rat mammary tumours.[40] Alternatively, the decreased PE/PC ratio could be uniquely associated with decreased proliferation of C6 cells. This would be consistent with a previous study of C6 cells in bioreactor cultures which showed a decrease in the PE/PC ratio upon transition from log to stationary growth phases.[41] Further work is required to differentiate between these possibilities.

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