Metabolite Changes in BT4C Rat Gliomas Undergoing Ganciclovir-Thymidine Kinase Gene Therapy-induced Programmed Cell Death as Studied by $^1$H NMR Spectroscopy in Vivo, ex Vivo, and in Vitro*

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Programmed cell death was induced by HSV-tk gene therapy in rat BT4C glioma cells, and metabolite changes associated with cell damage were monitored in vivo by $^1$H NMR spectroscopy and ex vivo by high resolution magic angle spinning (HRMAS) $^1$H NMR, and in vitro in perchloric acid extracts of tumors. Metabolite concentrations, as quantified in vivo using water as an internal reference and in vitro in extracts, were correlated with cell density. The results showed that both in vivo and in vitro glycine and creatine concentrations followed volume-averaged cell density, whereas that of total choline-containing compounds was unaffected by a cell loss approaching 60%. Meanwhile, both saturated and unsaturated $^1$H NMR visible lipids increased. HRMAS $^1$H NMR spectroscopy of the tumor samples at 14.1 Tesla demonstrated the presence of nucleotide peaks from adenosine and uridine nucleotides in glioma samples ex vivo. The assignment of a doublet at 7.95 ppm to UDP was confirmed by spiking experiments of tumor extracts in conjunction with $^1$H and $^{31}$P NMR spectroscopy. HRMAS also resolved the choline-containing peak at 3.2 ppm in vivo into resonances from choline (3.20 ppm), phosphocholine (3.22 ppm), glycerophosphocholine (3.24 ppm), and taurine (3.26 ppm). These resonances were uncorrelated with temporal progression through programmed cell death. Our results show that $^1$H NMR-detected lipids and some of the small molecular weight metabolites respond to gene therapy. However, the choline-containing compounds are unaffected by severe decline in cell density. The latter observation supports the idea that triacylglycerols, rather than membrane phospholipids, are the key components of $^1$H NMR visible lipids, and it also casts doubt on the validity of resonance of choline-containing compounds as a diagnostic marker of programmed cell death in vivo.

Programmed cell death (PCD) involves a cascade of biochemical processes in an ATP-dependent manner, and the process is associated with substantial morphological alterations in the cell interior before phagocytosis (1–3). Recent evidence from cell culture studies points to a number of characteristic metabolic perturbations appearing in the early phase of PCD. These include affections of intermediary metabolism, such as accumulation of glycolytic intermediates fructose 1,6-biphosphate, dihydroxy acetone phosphate, and glycerol-3-phosphate because of inhibition of glyceraldehyde-3-phosphate dehydrogenase (4), retention of CDP-choline (5) as a result of inhibition of CDP-choline:1,2-diacylglycerol choline phosphotransferase (6), and collapse of NAD(H) levels (5). The severe decline in NAD(H), leading to inhibition of glycolysis, may result from activation of poly(ADP-ribose) polymerase in apoptotic cells (5). Inhibition of CDP-choline:1,2-diacylglycerol choline phosphotransferase leads to cessation of phosphatidylcholine biosynthesis (6), and it has been proposed that this might be one of the mechanisms explaining accumulation of $^1$H NMR lipids into cells undergoing apoptosis (7, 8). Interestingly, evidence has been put forward to suggest that inhibition of phosphatidylcholine biosynthesis per se might induce PCD (9). Furthermore, cell studies have indicated altered phospholipid metabolism (10) and intracellular acidification in the early phase of PCD (5).

Both in cell cultures in vitro (7, 8) and tumors in vivo (11, 12), PCD has been shown to lead to accumulation of $^1$H NMR visible lipids. However, very little is known about the behavior of low molecular weight metabolites in vivo during ongoing PCD. Because a number of chemotherapeutic drugs are known to kill cancer cells by inducing apoptosis (2), information on low molecular weight markers of PCD would be crucial for the assessment of treatment responses in a clinical setting by $^1$H NMR spectroscopy (13). In the present study, we have monitored the non-lipid metabolite levels in rat BT4C gliomas during gene therapy-induced PCD in vivo in a quantitative manner as well as determined the metabolite concentrations in acid extracts of the tumor samples. Ex vivo high resolution magic angle spin-

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† The abbreviations used are: PCD, programmed cell death; HRMAS, high resolution magic angle spinning; GCV, ganciclovir; TR, repetition time; TE, echo time; STEAM, stimulated echo acquisition mode; SW, sweep width; MAS, magic angle spinning; CPMG, Carr-Purcell-Meiboom-Gill; PCA, principle component analysis; Glx, glutamate + glutamine; CHO, choline-containing compounds.
**EXPERIMENTAL PROCEDURES**

**Animals—** BT4C gliomas transfected with viral HSV-tk gene were induced by implanting 10^5 HSVG-tk cells in 5 μl of Opti-MEM to a depth of 2.5 mm into the corpus callosum of female BDIX rats weighing 180–250 g, as described previously (14). Rats in the treatment group were injected with GCV (25 mg/kg, intraperitoneal, twice daily) for the duration of the study. Brains were funnel-frozen in situ as described by Ponten et al. (15). The brains were sectioned, frozen, and tumor tissue was collected from a 100 mg piece of cryo-preserved tissue for in vivo 1H NMR spectroscopy or for extraction of acid-soluble metabolites with standard perchloric acid procedure. All animal experiments were performed according to the guidelines approved by the Ethical Committee of the National Laboratory Animal Center (Kuopio, Finland).

**NMR—** For MRI, rats (n = 15; 3 control rats and 12 treated animals) were anesthetized with 0.8–1.0% halothane in N_2O/O_2 (7:3) and the core temperature of animals maintained at 37 °C using a heated water blanket. MRI was performed in a horizontal 4.7-T magnet (Magnex Scientific Ltd, Abington, UK), equipped with actively shielded field gradients (Magnex Scientific) interfaced to a Varian ^1H NMRNOVA console (Varian, Inc., Palo Alto, CA). A quadrature surface coil (Highfield Imaging, Minneapolis, MN) was used in transmit/receive mode. Rats were fixed in a custom-built head holder using a mouth bar and ear pins, and tilted to an angle corresponding to a stereotactic rat brain atlas (16). Total imaging time for each animal was typically 80–90 min.

Tumor volumes were determined from T_2-weighted multi-slice spin-echo images (pulse repetition time (TR), 2.5 s; echo time (TE), 90 ms; field of view, 35 mm; matrix size, 256 × 128; 2 scans/line and contiguous slices of 1-mm thickness) (14). Absolute diffusion coefficient images were obtained using a spin-echo sequence (TR, 1.5 s; TE, 55 ms; matrix size, 128 × 64; slice thickness, 1.5 mm) and an adiabatic BIR-4 focusing pulse (17) with four bipolar gradient pairs in each direction. This achieves weighting by the trace of the diffusion tensor (D_{av} = [1–3] Trace D) in a single acquisition (18). Data from three acquisitions with different diffusion weighting, possessing b-values between 70 and 1420 s/mm^2, were used to calculate the absolute D_{av} images. A stimulated echo acquisition mode (STEAM) pulse sequence (TR, 3 s; middle delay period, 30 ms; TE, 2 ms; sweep width (SW), 2.5 kHz; 2000 data points) incorporating an outer volume saturation, a variable pulse power and optimized relaxation delay (YAPOR) water suppression scheme, and asymmetric excitation pulses (19) was used for quantification of tumor metabolites in vivo. A voxel was placed within the solid tumor according to the multi-slice T_2-weighted localized image, and the magnetic field within the voxel was optimized by shimming using the FASTMAP routine (20). In some animals, 1H NMR spectra were also acquired from the same voxel using the localization by adiabatic selective refocusing (LASER) method as described previously (21) (TR, 8.8 s; TE, 32 ms; SW, 2.5 kHz; 13,000 data points). Metabolite concentrations were quantified from STEAM spectra using non-suppressed water peak as a reference (0.787 kg/kg of fresh untreated BT4C tumor tissue) (22), corrected for T_2 saturation effects because of water accumulation during PCD, using the T_2 values previously reported (22).

**1H NMR Spectroscopy—** NMR spectroscopy, samples of tumor tissue (1–5 mg in wet weight) were placed into the zirconium oxide MAS rotor alongside 10 μl of D_2O (deuteron lock reference) containing 10 mM trimethylsilyl propionic acid (chemical shift reference). HAMAS 1H NMR spectra were acquired using a Bruker 600 MHz Avance spectrometer interfaced with a high resolution MAS probe (Bruker GmBH, Rheinstetten, Germany) at +4 °C using a conventional solvent suppressed pulse sequence based on the nuclear Overhauser spectroscopy pulse sequence to suppress baseline artifacts resulting from B_0 and B_1 field inhomogeneities (relaxation delay = π/2 – τ_1 – π/2 – τ_2 – τ_1 – π/2 – τ_2 TR, 2 s; SW, 10 kHz; 32,000 data points, solvent suppression applied during the preparation time (τ_1) of 4 μs and mixing time (τ_m) of 150 μs; 5 kHz spinning rate). Spectra were also acquired with a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence using a T_{E} of 40 ms (spin echo delay, 500 μs; total number of spin echoes, 40; other parameters identical to those described for the nuclear Overhauser spectroscopy presaturation pulse sequence).

Neutralized perchloric acid extracts were freeze-dried and samples dissolved in D_2O containing 20 μM trimethylsilyl propionic acid for chemical shift and concentration reference. High resolution pulse-acquired 1H NMR spectra using presaturation solvent suppression during relaxation delay (TR, 12.7 s; SW, 6 kHz, 16,000 data points) were collected at 500 MHz using a Bruker Avance spectrometer at 20 °C. The same instrument was used to acquire proton-decoupled 31P NMR spectra (TR, 2.4 s; SW, 40 kHz; 16,000 data points). To assign peaks tentatively identified as "nucleotides" in the ^1H and ^31P NMR spectra, some extracts were spiked with UDP, CDP, and their derivatives.

In vivo spectra were analyzed in the time domain using JMRUI software (web site: carbon.uab.es/mrui), in vitro extract spectra with PERCH software (www.perchsoftware.com), and the ex vivo spectra in the frequency domain using XWINNMR (Bruker GmBH). For metabolite quantification in vitro, the following chemical groups were used: a CH at 3.74 ppm for glutamate; a CH at 3.61 for myo-inositol; a CH_2 at 3.55 for glycine; a CH_3 at 3.42 for taurine; a (CH_3)_3 at 3.23, 3.21, and 3.20 ppm for glycerophosphocholine, phosphocholine, and free choline, respectively; a CH_3 at 3.03 ppm for creatine + phosphocholine; and a CH_3 at 1.47 ppm for alanine.

Student’s unpaired t test was used for statistical analysis of results. Linear regression analysis was used to estimate the significance of correlation between cell density and metabolite concentrations both in vivo and in vitro.

**Principal Component Analysis of ex Vivo Data—** To investigate the non-lipid metabolite changes that accompanied PCD in the tumors, high resolution MAS 1H NMR CPMG spectra (TE, 40 ms) were investigated using PCA. Spectra were converted into a data-reduced numeric format using automated integration across 0.04-ppm regions between 0.2 and 4.2 ppm as described previously (23). After global normalization, in which each integral region was represented as a ratio to the total integral across the 0.2–4.2 ppm region and mean centering of the each variable, the resultant data set was investigated using the PCA approach (SIMCA software; Umetrics, Umea, Sweden). The principal component representations of variation across the data set were then investigated in terms of clusterings relating to tissue type and progression through PCD. This process was also repeated for in vivo and in vitro 1H NMR spectra.

**Histology—** A satellite group of rats (n = 14; 2 control rats and 12 treated animals) were sacrificed by CO_2 and transcardially perfused with phosphate-buffered saline for 10 min (30 ml/min) followed by 4% paraformaldehyde in 0.1 mM phosphate buffer, pH 7.4, for 12 min (30 ml/min). The fixed brains were removed from the skull, rinsed in phosphate-buffered saline, and embedded in OCT medium (Bayer Corp., Emeryville, CA) for cryosectioning. Nissl staining was used to reveal the extent of cell damage in the sections (20-μm slices; 1:15 sections counted) as well as for quantitative cell counting as described previously (24). The adjoining sections were stained for terminal deoxynucleotidyl transferase dUTP nick-end labeling (ApopTag Plus; Oncor, Emeryville, CA) for apoptotic nuclei, using a methyl green counterstain. Apoptotic nuclei were counted in tumor tissue from arbitrarily chosen high power fields (>20; AX-70 microscope; Olympus, Tokyo, Japan).

**RESULTS**

Typical absolute distribution images through the center of tumor are shown from untreated and treated rats (Fig. 1, A and B). It was evident that D_{av} had increased by day 4 of GCV treatment in the center of tumor mass, and this was followed by a decline in the tumor volume commencing between days 4 and 6 (not shown). Histological sections showed substantial cell loss and scar formation (Fig. 1, compare C and D) starting from the center of the tumor volume, consistent with the patterns of D_{av} changes. Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining revealed substantial increase (>4-fold) in apoptotic nuclei by day 4. The average cell density was 178 ± 10 × 10^3 cells/mm^2 in non-treated tumors. The cell density was 183 ± 13, 154 ± 14, 109 ± 4, and 71 ± 2 × 10^3 cell/mm^2 in days 2, 4, 6, and 8 of treated tumors, respectively; day 6 and 8 values were significantly less than that at day 0 (p < 0.01). These observations confirmed that gene therapy had induced PCD (14, 22), resulting in severe cell loss in the gliomas.

Water-suppressed 1H NMR spectra from BT4C in vivo are dominated by strong lipid signals arising from a CH_3/CH_2 of saturated lipids at 1.3 ppm and a CH_3/CH_2 of saturated
lips at 0.9 ppm, but peaks from -CH=CH- of polyunsaturated fatty acids at 5.4 ppm, CH=CHCH=CH of PUFAs at 2.8 ppm, and CH₃CH=CH of lipids at 2.0 ppm are also discernible (Fig. 2). STEAM ¹H NMR spectrum from a tumor in vivo before GCV-treatment revealed well resolved peaks from phosphocreatine + creatine at 3.92 ppm, glutamate + glutamine (Glx) at 3.76 ppm, myo-inositol + glycine at 3.6 ppm, taurine at 3.43 ppm, choline-containing compounds (CHO) with a contribution from taurine at 3.23 ppm, and phosphocreatine + creatine at 3.03 ppm (Fig. 2). All these peaks were also visible in LASER spectra (Fig. 2, C and D). Short TE STEAM ¹H NMR spectroscopy was used to quantify metabolites in absolute terms, and the LASER method was used to qualitatively confirm the peak assignments from spectra with slight T₂ filter, yet with a higher signal-to-noise ratio compared with the STEAM sequence at similar TE. It should be noted that in the downfield part of the spectra beyond 6 ppm, no resolvable resonances were seen in vivo even after summing several proton spectra from different tumors to improve signal-to-noise ratio (not shown). The inserts of both STEAM and LASER spectra show the qualitative time-dependent changes in the metabolite peaks upon GCV treatment (Fig. 2, B and D). The absolute metabolite concentrations from STEAM spectra are plotted against cell density (Fig. 3). Although the concentrations of myo-inositol + glycine, taurine, and creatine showed a significant negative slope (Fig. 3, A and B) along with cell count in tumors undergoing PCD, that of CHO remained zero (Fig. 3C). A decreasing trend in the slope of Glx was also evident but was not significant (Fig. 3B). At the same time, both unsaturated (indicated by CH=CH at 5.4 ppm) and saturated (from CH₃CH₂CH₂ at 1.3 ppm) lipids by ¹H NMR indicated positive slope in the tissue with reducing cell density (Fig. 3D), as previously reported after GCV treatment of BT4C glioma (11, 12). It should be noted that the peak at 1.3 ppm may have contributions from lactate, but even during high-resolution MAS spectroscopy, the contribution from lactate was found to be small (data not shown).

Metabolite concentrations in acid extracts of tumor specimens are plotted against cell count (Fig. 3, E–G). Typical ¹H NMR spectra from untreated and treated tumor extracts are shown (Fig. 4, A–C). The in vitro results indicate a decreasing trend (p < 0.10) in concentrations of glycine, creatine, and alanine (Ala) accompanying reducing cell density (Fig. 3, E and F). However, taurine (Fig. 3E), Glx (Fig. 3E), and all choline-containing compounds (Fig. 3G) showed virtually zero slopes. Data in Fig. 3G indicate that the phosphocholine/glycerophosphocholine ratio increased during treatment from 0.31 ± 0.06 in untreated tumors to 0.64 ± 0.04 (p < 0.02) by day 6 of treatment. However, the respective values at days 4 and 8 of 0.60 ± 0.15 and 0.48 ± 0.07 were not different from those determined in the control tumors. The slope of myo-inositol showed a positive trend during PCD (Fig. 3E), which was unexpected against the in vivo observation (Fig. 3A).

Ex vivo HRMAS ¹H NMR spectra from normal parietal cortex and BT4C gliomas at different treatment days are shown in Fig. 5. Because of the excellent chemical shift resolution obtained with MAS at 14.1 T, one can assign individual signals from CHO, including choline, phosphocholine, and glycerophosphocholine, as well as taurine (Fig 5, A–C). No correlation was found between metabolite resonance intensities and day of treatment using multivariate regression techniques, indicating that changes in non-lipid metabolites did not reflect the pro-
gression of PCD (not shown). PCA of CPMG spectra separated tumor and control tissue across PCs 1 and 2 readily but did not identify a trend associated with PCD (Fig. 6A). Using the loading plots of the PCA model formed, the spectral regions most responsible for distinguishing tumor and normal tissue were identified. The spectra of tumors had increased relative resonance intensities from \( \text{CH}_3 \) lipid, \( \text{CH}_2\text{CH} = \text{CH} \) lipids, and \( \text{myo} \)-inositol. Further examination of loading plots demonstrated that normal brain cortex was characterized by resonances from lactate, \( \text{N} \)-acetyl aspartate, glutamate, and creatine (Fig. 6B). Repeating this analysis for spectra from extracts of tumors and in vivo spectra using the STEAM sequence, again the biggest discriminatory factor was the presence of NAA, creatine, and glutamate in control tissue. No metabolic trend associated with PCD was detected for non-lipid metabolites (not shown).

The downfield part of the HRMAS spectra revealed numerous peaks in tumors regardless of the treatment time that were not seen in the samples from normal brain. These peaks were more evident in CPMG spectra and were assigned to the nucleotides ATP and either UDP or CDP (Fig. 5, D–F). Peaks with same chemical shifts were also seen in the downfield part of acid-extracted tumors (Fig. 4, A and B). To determine whether the tumors contained uridine or cytosine metabolites, \( \text{P} \) NMR spectroscopy was performed on the aqueous tissue extracts of the tumors (Fig. 4, D and E), demonstrating the presence of ATP and UDP in the tumors. The assignment of the latter metabolite was confirmed by spiking experiments using both

![In vivo STEAM (A and B) and LASER (C and D) spectra from rat brain BT4C gliomas.](https://example.com/in_vivoSteam.png)

**Fig. 2.** In vivo STEAM (A and B) and LASER (C and D) spectra from rat brain BT4C gliomas. Water-suppressed \( \text{H} \) NMR spectra from the tumor volume presellected by \( T_2 \)-weighted MR images were acquired using STEAM (A–B) or LASER (C–D) methods. \( \text{mL} \), myo-inositol; \( \text{Lac} \), lactate; \( \text{Cr} \), creatine. \( \text{CHO} \) lipid peaks are assigned according the chemical groups giving rise to the resonance. B and D, the spectra are referenced to a fixed height of \( \text{CHO} \) peak. Day 6, Day 2, etc., refer to days 0 through 8 of GCV treatment.
FIG. 3. Absolute metabolite concentrations plotted against cell density in BT4C gliomas. Absolute metabolite concentrations (in micromoles per gram of tissue water) in vivo (A–D) were determined from STEAM spectra using endogenous water as an internal reference. Metabolite concentrations were also determined from acid-extracted tumor specimens in vitro by 1H NMR spectroscopy (E–G) as described under "Materials and Methods." Cell densities were determined at corresponding treatment day from Nissl-stained sections as described under "Materials and Methods." Linear regression analysis gave correlation coefficients and p values for in vivo peaks as follows: taurine, R² = 0.78, p < 0.05; myo-inositol (mI) + Gly, R² = 0.86, p < 0.03; creatine (Cr), R² = 0.76, p < 0.05; Glx, R² = 0.62, p < 0.12; CHO, R² = 0.009, p < 0.9; 1.3 ppm, R² = 0.94, p < 0.01; 5.4 ppm R² = 0.91, p < 0.02. The respective correlation coefficients and p values for in vitro metabolites were as follows: taurine, R² = 0.58, p < 0.24; myo-inositol, R² = 0.83, p < 0.09; Gly, R² = 0.89, p < 0.06; creatine, R² = 0.83, p < 0.09; Glx, R² = 0.13, p < 0.63; free choline (fCHO), R² = 0.43, p < 0.34; phosphocholine (PC), R² = 0.052, p < 0.77; glycerophosphocholine (GPC), R² = 0.55, p < 0.25; Ala, R² = 0.84, p < 0.09.
Fig. 4. Typical $^1$H (A, B, and C) and $^{31}$P (D and E) NMR spectra from neutralized acid extracts of BT4C gliomas. In vitro $^1$H spectra from days 8 (A) and 4 (B) of GCV treatment and an untreated tumor (C) and $^{31}$P NMR spectra from day 8 (D) and an untreated glioma (E) were acquired as described under “Materials and Methods.” ml, myo-inositol; Lac, lactate; Cr, creatine; ANP, adenosine nucleotides; For, formate; H-tau, hypotaurine; Suc, succinate; Ace, acetate; β-OH-But, β-hydroxy-butyrate; Gl-3-P, glycerol-3-phosphate; PE, phosphoethanolamine; $P_i$, inorganic phosphate; GPE, glycerophosphoryl ethanolamine; PCr, phosphocreatine; GPC, glycerophosphocholine; PC, phosphocholine.
**DISCUSSION**

In the present study, we have used BT4C tumors undergoing ganciclovir-HSV-tk gene therapy as a model of PCD. This well documented model has characteristic metabolic changes associated with PCD, most notably a large increase in polyunsaturated fatty acids. Furthermore, in this model, we have recently demonstrated by immunohistochemical methods that monocyte-macrophage invasion of the tumors is minor, ensuring that the majority of metabolic changes detected are from PCD (25).

The present results show that subsets of 1H NMR detectable metabolites in vivo behave in a different fashion during PCD-induced cell loss in BT4C glioma. The first group of metabolites, comprising glycine, taurine, and creatine, decline in a time-dependent manner largely after the cell loss. The second group of metabolites, represented by CHO, stay unchanged despite reduced cell density until catastrophic collapse of viable cells. The third group of 1H NMR-detectable metabolites, represented by both saturated and polyunsaturated lipids, extensively increase during PCD despite severe cell loss (11). Thus, the behavior of 1H NMR visible lipids during PCD shows an inverse correlation to that of low molecular weight metabolites, such as glycine and creatine. 1H NMR lipid changes in PCD have attracted wide scientific interest, and these changes have been demonstrated both in cells in vitro (7) and tumors in vivo (11, 12). The 1H NMR lipid observation is accounted for by accumulation of triacylglycerols (8), free fatty acids, and cholesterol esters into the tumors leading to increase in intracellular and other lipid vesicles (11).

In this study we have used tumor acid extracts to assess the correlation between in vivo and in vitro 1H NMR. However, it is important to realize that a direct comparison of in vivo and in vitro metabolites against tumor cell count is not straightforward, because in vitro extracts represent only part of the total tumor volume, whereas the cell counts are from the global tumor volume. Despite this fact, in vivo and in vitro 1H NMR spectroscopic data generally show a good degree of consistency for several metabolites, such as CHO, glycine, and creatine. In contrast, concentration plots of myo-inositol and Glx against cell density show discrepancies between the in vivo and in vitro figures. The reason for these discrepancies is not directly evident from our results. However, factors associated with sample preparation, such as post mortem metabolism and acid extraction, may potentially alter concentrations of metabolites. For instance, it has been shown that acid extraction yields greater glutamate concentration from guinea pig cerebral cortex than...
is detected by $^1$H NMR spectroscopy in situ, possibly as a result of an NMR invisible pool of glutamate (26). Furthermore, the very act of extraction biases the spectra toward metabolites most soluble in the extraction media, and thus small amounts of contamination from control tissue may affect the in vitro results to a greater extent than those obtained in vivo.

It has been shown that in long TE spectra from treated brain tumors, absence of discernible metabolite peaks or presence of lactate indicate only acellular tissue after necrotic cell death (13). Thus, the end stage of successful treatment is readily identifiable by $^1$H NMR. The present observations from rat glioma may have practical implications for the interpretation of in vivo $^1$H NMR spectroscopy of treated brain tumors as well. Our present and earlier results (11, 27) demonstrate that several dynamic $^1$H NMR spectroscopic changes may potentially indicate on-going cell death before the collapse of low molecular weight metabolites. The most prominent of these is seen as accumulation of lipids, particularly in polyunsaturated lipids (11, 12), reflecting both PCD-induced biochemical events (6) and altered NMR visibility of lipids (11, 28). Interestingly, ex vivo $^1$H NMR has shown strong lipid peaks from acellular (“necrotic”) human brain tumor samples (29, 30). The present results examine the potential of high field quantitation of $^1$H NMR spectroscopy to detect ongoing cell death by the correlation of certain low-molecular weight metabolites with tumor cell density during PCD. Previously, it has been shown in a human brain tumor study that the water apparent diffusion coefficient and in vivo CHO concentration correlate with cell density (31).

The present data strongly argue that decline in CHO in vivo is a $^1$H NMR marker of advanced stage of PCD only. This is a very important observation, because in clinical long TE $^1$H NMR of tumors, choline-containing metabolites possess one of the few visible peaks. In treated human brain tumors, the $^1$H NMR voxels showing either no metabolite peaks (13) or only lactate (32) apparently correspond to necrosis. Consistent with this observation, Kizu et al. showed that in the brain tumors after radiosurgery metabolites become undetectable in the necrotic tissue. In some cases, however, macroscopically necrotic tissue showed strong signals from both lipids and CHO (33). Severe decline in CHO has also been observed in tumors responding to radiation (34). Previous BT4C glioma gene therapy studies using STEAM $^1$H NMR spectroscopy with TE $>$20 ms at 9.4 T have noted a decline in CHO in vivo (11, 27). These two technical differences with respect to the present study may explain the discordance in $^1$H NMR CHO behavior in advanced PCD in vivo.

Two plausible possibilities explain our current observation. First, accumulation of CDP-choline during PCD may maintain the choline-containing peak despite significant cell loss. It was recently demonstrated by multinuclear NMR analyses of leukemia HL-60 cells (5) that this intermediate of phosphatidylcholine biosynthesis accumulates during PCD in vitro. However, we found no support for this hypothesis to explain the current observations either from $^1$H or $^{31}$P NMR of extracted tumors. The observation of CDP-choline changes in vivo using the choline resonance at $\approx$3.2 ppm in vivo is unlikely to be practical. CDP-choline has a singlet at 3.23 ppm, thus overlapping with the CHO peak in vivo and with phosphocholine in vivo in the BT4C tumor extracts and ex vivo in intact tumors. This resonance is nearly co-resonant with phosphocholine and glycerophosphocholine, and the triplet from taurine also contributes to this set of resonances. This is readily observable using HRMAS $^1$H NMR spectroscopy ex vivo and indicates that previously reported changes of choline-containing metabolites by short TE $^1$H NMR, correlating with progression of PCD, are likely to be confounded by changes in the taurine triplet at 3.26 ppm. Both in vivo and in vitro spectra detected decreasing taurine concentrations, and this decrease offset any increase in CDP-choline detected in vivo as part of the combined choline-containing resonance.

Second, an alternative explanation for the maintenance of resonance intensity from choline-containing metabolites in vivo at the end stage of PCD despite cell loss may be associated with a repartitioning of the metabolites between NMR invisible and visible environments. Even under MAS conditions used here, spinning speeds were too slow to remove the effects of the large dipolar couplings produced between lipids in cell membranes (35); thus, $^1$H NMR-detected CHO are likely to be non-membrane bound. Previously, we have detected increases in CHO after cell membrane disruption in cultured hepatocytes, with the increased choline-containing metabolite resonance arising from membrane phosphocholine being redistributed to the cytosol (36). It is conceivable that during the end stage of PCD, the CHO resonance intensity is probably associated with cell membrane disruption and leakage of choline-containing metabolites into the cytosol and extracellular fluid. This would explain why the CHO resonance remains fairly constant in terms of cell count, whereas some other low molecular weight metabolites decrease in resonance intensity.

Using HRMAS spectroscopy in conjunction with the CPMG pulse sequence, we detected a doublet at 8.01 ppm and two broader resonances at 6.08 and 6.13 ppm. These resonances are consistent with C6, C1, and C5 resonances on either uridine or cytosine bases. The detection of nucleotide resonances in proliferating cell cultures has previously been reported both using cell extracts of lymphocytes and HRMAS $^1$H NMR spectroscopy of intact endometrial cells (37, 38). Furthermore, in the latter study, tamoxifen treatment, a known selective estrogen receptor modulator, increased the concentration of nucleotides detected. Examination of the tumor tissue extracts with $^{31}$P NMR spectroscopy indicated that these resonances were from UDP. Accumulation of this nucleotide in glioma may result from metabolic pathways common with glial cells, because UDP-glucose is used to control glycogen turnover in cells that store glycogen, such as astrocytes (39). Alternatively, the pool of UDP may signify the increased synthesis of nucleotides for DNA synthesis necessary for replication of proliferative cells. Because uridine is a component of RNA, it may accumulate in the cell, whereas the other nucleotides are used to replicate DNA. This provides an explanation for the detection of relatively large concentrations of uridine containing metabolites in lymphocytes (37), testes (40), immortalized cell lines (38), and, in this study, glioma.

In conclusion, unlike the well documented increases in saturated and unsaturated fats during PCD, non-lipid metabolites seem poor indicators of the progression of PCD. Cell death is characterized largely by a decrease in some metabolites, consistent with a reduction in cell count within the tumors. Although nucleotides, including relatively large amounts of UDP, were detected in tumors after treatment, their quantities remained constant. Thus, the monitoring of saturated and unsaturated lipid resonances remains the most informative marker of cell death in BT4C tumors during HSV-tk gene therapy.

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**Metabolite Changes in BT4C Rat Gliomas Undergoing Ganciclovir-Thymidine Kinase Gene Therapy-induced Programmed Cell Death as Studied by $^1$H NMR Spectroscopy in Vivo, ex Vivo, and in Vitro**

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