Human glioma cells exhibit marked sub-acute reductions in oxygen consumption and TCA cycle activity during temozolomide-induced apoptosis

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Introduction: Most chemotherapeutics function by inducing apoptosis in tumor cells. One of the early changes that occurs during apoptosis is release of cytochrome-c from the space between the inner and outer mitochondrial membranes (1). In the cytoplasm, cytochrome-c plays a central role in the apoptotic cascade; however, it can no longer perform its normal functions in oxidative phosphorylation. We hypothesized that human glioma cells treated with temozolomide (TMZ), a new alkylating agent for treatment of brain tumors, would exhibit disrupted oxidative metabolism that could be detected with ^{13}C NMR. To examine this hypothesis, cells were studied with an artificial tumor method developed in our lab, which allows apoptotic cells to be monitored under well-defined conditions (2). ^{13}C NMR spectra were acquired continuously, while the tumor was perfused with culture medium containing [1,6-$^{13}C_2$]glucose.

Materials and Methods: SF188 cells (human glioma grade 4, Brain Tumor Research Center, UCSF) were grown with DMEM medium (supplemented with 10% serum and 50 μg/ml gentamicin) in porous collagen microcarriers (Hyclone, Logan, UT). These microcarriers had a mean diameter of 200 μm when fully hydrated and were used in combination with non-porous polystyrene spheres (1:1 volume ratio) inside a 20-mm NMR tube (2). The cells were sustained at physiologic conditions (37 °C, pH = 7.2, dissolved oxygen = 0.2 mM) with a system constructed in our laboratory (3). NMR spectra were acquired with a 9.4T spectrometer (Varian, Palo Alto, CA). ^{31}P spectral parameters were: 60° pulse width, 1000 ms repetition time, 4096 points, and 15000 Hz spectral width. ^{13}C spectra were acquired with 60° pulses, a repetition time of 1200 ms, 4096 points, 25000 Hz spectral width and ^{1}H bi-level WALTZ-16 decoupling. Cells were initially fed DMEM with 10 mM un-enriched glucose while background spectra were acquired. Subsequently, the un-enriched medium was completely replaced with DMEM containing 10 mM [1,6-$^{13}C_2$] glucose (Cambridge Isotopes, Andover, MA). Absolute intracellular concentrations were calculated as described previously (3). Oxygen consumption was determined continuously with polarographic oxygen probes located upstream and downstream of the tumor (4). ^{13}C measurements were performed for three independently prepared artificial tumors. In parallel studies, cells grown inside microcarriers were treated with TMZ and examined with a standard TUNEL assay for apoptosis (BD Biosciences, Palo Alto, CA). Endonuclease-cleaved DNA was labeled with fluorescein and detected with fluorescence confocal microscopy (Biorad, Hercules, CA).

Results: Approximately 12 days after microcarrier inoculation, the total viable cell number (estimated from ^{31}P NMR-detected NTP levels (4)) in the artificial tumor was ~8 x 10^8. A typical ^{13}C spectrum acquired during infusion with 10 mM [1,6-$^{13}C_2$]glucose is shown in Figure 1. Strong resonances were detected for C-1 and C-6 of glucose and C-3 of lactate and alanine. Analysis of the perfusate with ^{13}C NMR demonstrated that these signals were largely extracellular. In addition, significant levels of label were detected in C-4, C-3 and C-2 of intracellular glutamate. For 15-min spectra processed with 5-Hz exponential line broadening, the SNR for C-4 and C-2 of glutamate were 35:1 and 6:1, respectively. The C-3 glutamate resonance was a triplet due to coupling with C-4 of glutamate (3). A small amount of label was also detected in resonances associated with –CH_2- groups of mobile lipids (30.4 and 29.8 ppm) and glycolytic intermediates (63 to 68 ppm). Following the ^{13}C infusion, cells were treated twice with TMZ (130 μg/ml with a delay of 24 h between treatments). NTP levels and oxygen consumption began to decline approximately 12 hours after the second treatment. The decline in oxygen consumption was more rapid than the decline in NTP. Four days after the second TMZ treatment, the tumor was again perfused with medium containing 10 mM [1,6-$^{13}C_2$] glucose. No labeling was detected for any intracellular resonances except a very small amount at C-4 of glutamate. The time course for this resonance is shown in Figure 2. On an NTP normalized basis, labeling was markedly reduced relative to that observed before treatment. The oxygen consumption rate was reduced by more than 95% and was below the detectable limit. Similarly, the rate of labeling in C-4 glutamate (per unit of NTP) was reduced by 85%. Although glucose consumption and lactate formation proceeded at a relatively high rate, both were only 50% of the rates observed prior to treatment (per unit of NTP). No [3-^{13}C]alanine was detected. TUNEL analysis demonstrated that 2 days after the second treatment, ~10% of cells in the microcarriers contained endonuclease cleaved DNA. This value increased to ~30% 3 days post-treatment.

Discussion: Glutamate is a key reporter molecule for TCA cycle labeling (5). [3-^{13}C]alanine is formed from [3-^{13}C]pyruvate by transaminases in mitochondria. The marked reduction in labeling for glutamate and alanine and the lack of oxygen consumption indicate that mitochondrial function was disrupted sub-acutely by TMZ. The reduction in lactate formation was much smaller on a percentage basis; however, it demonstrates that cytoplasmic metabolism is also disrupted sub-acutely following therapy. Because endonuclease cleavage of DNA occurs relatively late in apoptosis, the finding that only 30% of cells were stained in the TUNEL assay was not surprising. Many cells may have released their cytochrome-c into the cytoplasm, but had not yet initiated DNA cleavage. Direct detection of cytochrome-c release will be studied in future work to elucidate the time course for this important change following TMZ treatment.

Conclusions: Our results indicate that ^{13}C NMR can be used to detect changes in oxidative metabolism of human glioma cells following treatment with TMZ. This finding may help to advance the use of ^{13}C MRS methods (e.g. ^{1}H-detected ^{13}C MRS) for clinical monitoring of apoptosis in human gliomas.

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References:

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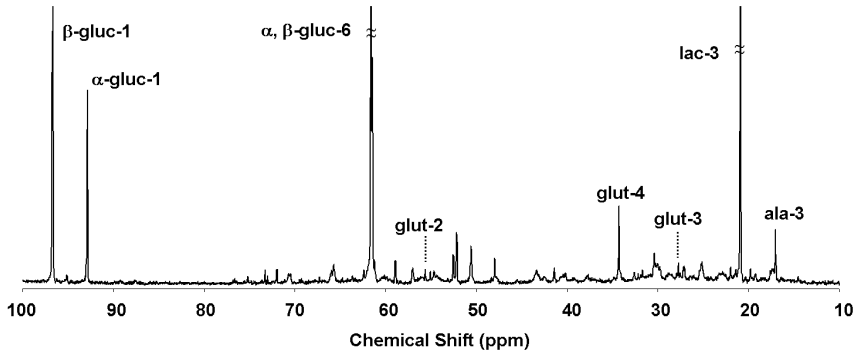

Figure 1. ^{13}C spectrum of untreated SF188 cells during infusion of [1,6-$^{13}C_2$]glucose. This spectrum was obtained in 30 minutes.

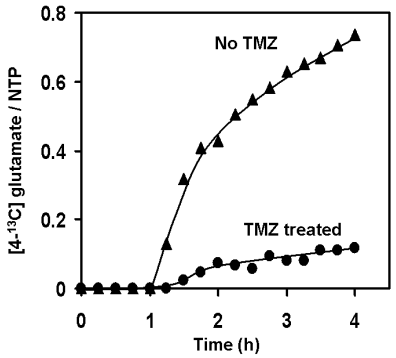

Figure 2. ^{13}C labeling in C-4 glutamate per unit of NTP is markedly reduced 4-days after treatment with TMZ.