317g Human Glioma Cells Undergoing Chemotherapy-Induced Apoptosis Exhibit Marked Reductions in Intracellular Phosphocholine and Phosphocreatine

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Introduction: In current clinical practice, the response of human brain tumors to therapy is generally monitored with magnetic resonance imaging (MRI) methods that detect changes in tumor morphology or blood flow. These changes generally occur slowly and often many weeks can pass before they are detectable. Without a reliable means for detecting response early during the course of treatment, patients can be subjected to harsh, ineffective therapeutics for an unnecessarily long period of time.

^{31}P NMR spectroscopy can detect biochemical changes in treated tumors that often precede morphological changes (1). Rapidly proliferating tumor cells frequently contain high levels of free phosphomonoesters such as phospho-choline and phospho-ethanolamine. These compounds are precursors for phosphoglycerides, which are a major subclass of phospholipid found in cellular membranes. Proliferating tumor cells also frequently contain elevated levels of phosphocreatine, which is used for storage of metabolic energy. In response to successful chemotherapy, tumor cells generally undergo apoptosis. This process produces marked changes in cellular energetics and membrane structure as the cytoplasm and nucleus condense into apoptotic bodies. We hypothesized that human glioma cells undergoing apoptosis would exhibit marked, selective changes in phosphomonoester and phosphocreatine levels.

To examine this hypothesis, metabolically active human glioma cells were studied non-invasively with ^{31}P NMR spectroscopy *ex vivo*. The cells were grown in a perfused artificial tumor, constructed of porous collagen and non-porous polystyrene microspheres. The tumor was specifically designed to retain apoptotic cells, which are often lost in perfusion experiments as they detach from their growth surfaces. The results may be useful for the design of new clinical methods that are much more sensitive to early therapeutic response than current MRI methods.

Materials and Methods: SF188 cells (human grade 4 glioma) were obtained from the UCSF Brain Tumor Research Center (San Francisco, CA). They were grown in Dulbecco's Modified Eagles Medium (DMEM) that contained 25 mM glucose, 6 mM glutamine, 50 mg/ml gentamicin sulfate and 10% fetal bovine serum. Pre-sterilized porous collagen microcarriers (200 mm diameter, Hyclone, Logan, UT) were inoculated with freshly trypsinized cells at a ratio of ~10^7 cells/hydrated gram of collagen. The cells were allowed to grow inside the microcarriers for 9 to 11 days. To construct the artificial tumor, the microcarriers were mixed at a 1:1 volume ratio with solid polystyrene micro-spheres and tightly packed into a 20-mm NMR tube (2). The polystyrene helped to limited compression of the porous collagen during perfusion (12 ml/min). 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. Oxygen consumption was determined continuously with polarographic oxygen probes located upstream and downstream of the tumor. Experiments were conducted with either one 160 mg/ml (N=1) or two 125 mg/ml (N=4) doses of TMZ (with a 24-h delay between treatments). 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 within the microcarriers with fluorescence confocal microscopy (Biorad, Hercules, CA). **Results:**

Compounds detected with high signal to noise included phosphocholine (PCh), inorganic phosphate, gycerophospho-choline (GPC), phosphocreatine (PCr), nucleoside triphosphates (NTP, the sum of ATP, CTP, GTP, TTP and UTP) and diphosphodiesters (DPDE, which are the sum of NAD(H) and uridine diphospho-sugars). The total number of viable cells in the tumor was ~4 x 10^8 prior to treatment (estimated from the NTP level). In response to a single dose of TMZ (160 mg /ml), NTP and PCr levels continued to increase for an additional 40 hours and subsequently declined linearly. PCr reached undetectable levels ~85 h after treatment, which coincided with cessation of oxygen consumption. In contrast, PCh levels began to decline within 25 hours of treatment and were nearly undetectable 65 hours later. The GPC and DPDE levels were the slowest to respond to therapy. The percentage reductions were largest for PCr (100%) and PCh (87%); smaller reductions were observed for GPC (39%) and DPDE (57%).

TMZ has a very short half-life in blood and culture medium (~1.5 hr) and is normally administered once every 24 hours for five successive days. To examine the effects of repeated doses, an artificial tumor containing a much higher level of cells, ~9 x 10^8, was treated with two doses of TMZ (125 mg/ml, 24 h between treatments). The results were qualitatively similar to those described above. The percentage reductions were largest for PCr (100%), and PCh (83%); significantly smaller reductions were observed for GPC (58%) and DPDE (54%).

Confocal microscopy results indicated that for the tumor treated with 160 mg/ml TMZ, no apoptotic cells were detected over the first 24 hours, but nearly half were apoptotic by 72 hours. For the tumor containing a much higher cell number and treated with twice with 125 mg/ml, apoptosis developed more slowly; approximately 20% of cells were apoptotic 72 hours after the second treatment.

Discussion: The reduction in PCh is consistent with the findings for other tumor types, including breast and prostate, in response to therapy (1,5). However, the reduction was not associated with an increase in GPC levels as had been reported with anti-mitotic agents (6). Although changes in PCr are less commonly reported in tumors, this metabolite was the most sensitive indicator of response for SF188 cells. Complete depletion occurred when oxygen consumption ceased, while significant levels of NTP were still present. These findings are consistent with the belief that PCr is used to store excess metabolic energy. **Conclusions:** The selective reductions in PCh and PCr observed in response to therapy may be useful for the design of clinical methods for detecting apoptosis. In future work, we will conduct more detailed studies to quantify the relationship between reductions in the levels of these two compounds and specific events in the apoptotic cascade.

References:

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