Cyclophosphamide Treatment Modifies Tumor Oxygenation and Glycolytic Rates of RIF-1 Tumors: 13C Magnetic Resonance Spectroscopy, Eppendorf Electrode, and Redox Scanning

Harish Poptani,1 Navin Bansal,1 Walter T. Jenkins,2 Dana Blessington,4 Anthony Mancuso,1 David S. Nelson,1 Michael Feldman,3 Edward J. Delikatny,1 Britton Chance,4 and Jerry D. Glickson1

Departments of 1Radiology, 2Radiation Oncology, and 4Pathology and 4Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania

ABSTRACT

The effect of cyclophosphamide (Cp) on the glycolytic rate of radiation-induced fibrosarcomas (RIF-1) was measured in vivo in C3H mice by following the production of [3,13C]lactate after tail vein infusion of labeled [1-13C]glucose. Cp administered i.p. at a dose of 300 mg/kg caused a significant drop in glycolytic rate 24 h after treatment (P < 0.01). This drop was accompanied by an increase in [C-3]/[C-4] glutamate ratio in perchloric acid extracts of the tumors, indicating an increase in the Kreb’s cycle activity. Treatment with Cp led to a significant decrease (P < 0.01) in tissue pH, measured in vivo with an oxygen Eppendorf electrode. Increases in NADH levels were also observed in rapidly frozen excised tumors examined by three-dimensional optical redox scanning. A significant decrease in tumor pH and an increase in the NADH levels are suggestive of an increase in oxygen consumption by these tumors after Cp treatment. Overall, these data indicate that the reduction in glycolytic rate of Cp-treated RIF-1 tumors is due to an increase in aerobic metabolism.

INTRODUCTION

As discovered by Warburg (1), tumors in general prefer glycolytic metabolism over oxidative phosphorylation to meet their energy demand. Increased aerobic and anaerobic glycolysis leads to accumulation of steady-state lactate levels in tumors that can be detected by in vivo proton magnetic resonance spectroscopy [MRS (2–9)]. Elevated lactate levels are detected due to an imbalance between lactate production (glycolysis) and clearance (wash out) and have been proposed as markers for tumor diagnosis (10) and poor prognosis (11, 12). However, many brain tumors do not exhibit elevated levels of lactate (13), probably due to efficient mechanisms of lactate clearance by the vasculature. A decrease in lactate level after successful therapy may serve as an indicator of early response to radiation (2, 6) and chemotherapy (3, 8). Although the exact mechanism for the drop in steady-state lactate after therapy is not clear, possible mechanisms may include improved perfusion, oxygenation, or alterations in activity of enzymes involved in glucose metabolism.

Because steady-state measurements of tumor lactate by 1H MRS do not provide an estimate of glycolytic flux, 13C MRS has been used to measure tumor glycolytic rates in vivo (4, 9, 14–20). The method involves intratumoral or systemic infusion of 13C-labeled glucose and measurement of labeling in key metabolites [glutamate for the tricarboxylic acid (TCA) cycle and lactate for glycolysis (4, 9, 14–20)]. Most tumors exhibit labeling of the [C-3] lactate, which results directly from glycolysis (4, 14, 16, 18–22), whereas other studies indicate labeling of both [C-3] lactate and [C-4] glutamate, reflecting various levels of both glycolytic and oxidative metabolism (9). When glucose (the substrate) and lactate (the end product of glycolysis) are the only isotopically labeled metabolites observed in vivo (i.e., when labeling of glycolytic intermediates is negligible), the 13C MRS data can be analyzed using a two-compartment model to estimate the glycolytic and lactate clearance rate constants (21).

Because steady-state levels of key metabolites are maintained by various homeostatic mechanisms that modulate rates of synthesis and clearance of these molecules, kinetic measurements of rates of production or clearance of these molecules should be more sensitive indices of the status of host tissues than the corresponding steady-state measurements. These considerations led to the conclusion that the glycolytic rate should be more sensitive than steady-state lactate measurements in monitoring the status of the tumor and detecting its response to tumor therapy. Therefore, in vivo 13C MRS was used to determine whether changes in glycolysis could be used to monitor response to cyclophosphamide (Cp) treatment in the RIF-1 tumor model. Changes in oxidative metabolism of treated and control tumors were monitored by high-resolution 13C MRS of tumor extracts and by measuring the glutamate pool size using high-performance liquid chromatography (HPLC). Changes in oxygen consumption rates were inferred from polarographic measurements of tumor oxygen tension and three-dimensional optical imaging of mitochondrial redox status.

MATERIALS AND METHODS

Tissue Culture. RIF-1 tumor cells were grown as monolayers using Waymouth's culture medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 10 mM HEPES buffer. The tumor cells were passaged between in vitro culture and in vivo tumors according to the protocol of Twentyman et al. (23).

Animals and Tumors. All animal procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Female C3H/HeN mice (National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD), approximately 6 weeks of age, weighing 18–20 g each, were used. Tumors were propagated s.c. by inoculating 2 × 104 cells in a 50-μl volume of Hank’s solution in the upper thigh. Animals were anesthetized with an i.p. injection of 0.1 ml of 50 mg/kg ketamine (Fort Dodge Animal Health, Inc., Fort Dodge, IA) and 5 mg/kg acepromazine (Fermenta Animal Health Co., Kansas, MO). Tumor size was measured with calipers in three orthogonal dimensions, and tumor volume was calculated with the formula of an ellipsoid using Eq. 1:

\[ V = \frac{\pi}{6} x y z \]

where x, y, and z are the length, breadth, and depth of the tumor.

Growth Delay Studies. Tumors were treated with a single dose of Cp (300 mg/kg, i.p.; Sigma-Aldrich) 2 weeks after cell inoculation. The difference in the time required to reach a predetermined volume (1000 mm3) for control (n = 11) versus treated (n = 5) animal groups defined the absolute growth delay.

Doppler Ultrasound Measurements. To observe the effect of hyperglycemia on tumor blood flow, 300 mm glucose was infused i.v. in three tumor-bearing animals using a double syringe pump (Harvard Apparatus, Inc., Hol-
HPLC Analysis. Aliquots of the perchloric acid samples used for high-resolution $^{13}$C MRS were used to determine the amino acid composition using HPLC. Samples were prepared with L-aminoadipate as an internal standard. The imaging was performed using a 15-12 MHz broad band transducer. An image was scanned before and after infusion of glucose. A bubble was observed in an approximately 0.2 mm was identified in the image plane, and spectral Doppler was recorded at an interval of every 2–5 min. Peak flow velocity was determined from each tracing.

MRS Experiments. MRS experiments were performed 2 weeks after tumor cell inoculation. Eight tumor-bearing mice were treated with Cp, whereas six animals were used as controls (sham-treated with saline). MRS measurements were made before treatment with Cp (pretreatment) and 24 h after the treatment. Control animals were studied at corresponding time points. MRS was performed using a 9.4T vertical bore magnet interfaced to a Varian Inova (Varian Inc., Palo Alto, CA) console. A double-tuned looped-gap resonator ($^{1}$H decoupled) $^{13}$C spectra was acquired using $^{1}$H decoupled $^{13}$C spectra. A 26 G Abbot-T catheter (Abbott Ireland, Sligo, Republic of Ireland) was placed in the tail vein of the tumor-bearing animal to infuse 300 mM $[^{1}-^{13}$C$]$glucose for 40 min with a double syringe pump (Harvard Apparatus, Inc.). The infusion rate was variably adjusted to maintain the blood glucose level at 17 ± 2 mm (24). The animal was positioned on top of a plastic cradle containing the slotted tube resonator such that the tumor was positioned inside the resonator. A rectal temperature, which was maintained at 37°C with warm air blown through the magnet bore. The magnetic field homogeneity was shimmed to a line width of less than 100 Hz at half height on the $^{1}$H water signal. Proton decoupling was performed using a WALTZ-16 pulse train with the nuclear Overhauser effect power being half of that used for frequency heating, proton decoupling was performed using a bilevel decoupling pulse sequence with a repetition time of 25,062 Hz.

Tumor Blood Flow by Doppler Ultrasound. In vivo tumor blood flow velocity was determined from each individual tumor and then averaging these values across the two-compartment model developed previously (21).

In Vitro NMR Experiments. Separate cohorts of tumor-bearing animals (treated ($n = 4$) and control tumors ($n = 3$)) were infused with $[^{13}$C$]glucose, and tumor tissues were excised and freeze-clamped in liquid nitrogen immediately after the 40 min glucose infusion protocol used for the in vivo experiments. Perchloric acid extractions from these tumor tissues were prepared as described elsewhere (25). Briefly, frozen tissues were ground under liquid nitrogen and homogenized in 6% ice-cold perchloric acid. The homogenate was centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant was neutralized to a pH of 7.0 ± 0.2 using 3 M KOH. The precipitate was removed by centrifugation, and the supernatant was lyophilized. The resulting powder was redissolved in 0.5 ml of D$_{2}$O, and the pD was adjusted to 7.0 ± 0.1. High-resolution proton-decoupled $^{13}$C MRS spectra were acquired using WALTZ-16 decoupling, 1-s-saturation period, 64,000 points, and a spectral width of 25,062 Hz.

HPLC Analysis. Aliquots of the perchloric acid samples used for high-resolution $^{13}$C MRS were used to determine the amino acid composition using HPLC. Samples were prepared with L-aminoadipate as an internal standard. The solution was centrifuged, and the pH was adjusted to 8.5–9.0 using KHCO$_{3}$. Amino acids were measured after pre-column derivitization with o-phthalaldehyde using an automated injector as described previously (26). After resolving the amino acids with reverse-phase chromatography, amino acids were detected using a McPherson Model FL-750BX fluorescence detector. Peak areas were integrated using Millenium software (Waters/Millipore, Milford, MA).

Measurements of Tumor Tissue Oxygenation. Tumor pO$_{2}$ measurements were performed on separate cohorts of animals (10 treated and 10 controls) with an O$_{2}$-sensitive needle electrode (KIMOC 6650; Sigma pO$_{2}$ Graphist; Eppendorf, Hamburg, Germany) as described previously (27).

Briefly, the electrode was moved through the tissue in steps of 0.4 mm. Each forward motion of 0.7 mm was immediately followed by a backward step of 0.3 mm to relieve tissue pressure. Local oxygen partial pressures (pO$_{2}$) were measured 1 s after the backward motion. Two tracks were measured from each tumor, generating 20–30 pO$_{2}$ values. Measurements were performed immediately after the glucose infusion was stopped. Negative values of pO$_{2}$ were set to zero for all of the experiments (28).

Fluorescence Imaging of Tumors. Tumor NADH levels were imaged using the methods described previously (29–31). Briefly, immediately after glucose infusion, tumor-bearing animals were immersed in precooled isopentane (−150°C) and transferred to liquid nitrogen (−196°C) 5 min later. Tumors, along with the underlying muscle, were then surgically excised, embedded in a mixture of ethanol-glycerol-water (freeze point, −30°C), and mounted at low temperature for three-dimensional surface fluorometric scanning. The frozen tumor sample was milked flat and imaged every 100 μm from the top surface to the bottom of the tumor. The light guide (fused silica, 50-μm core diameter) was stepped across the tissue surface at a fixed distance from the tissue surface (70 μm). The isotropic imaging resolution of the low-temperature scanning fluorometer was 80 μm. The fluorescent signals of oxidized flavoproteins (filters: Excitation (Ex), 440DF20; Emission (Em), 520DF40) and pyridine nucleotides (PNs; filters: Excitation (Ex), 365HT25; Emission (Em), 455DF70) were imaged for each depth of tumor. The scanning was performed at 128 × 128 steps to cover a region of 1.024 × 1.024 cm$^{2}$. The fluorescence signal was automatically digitized and recorded on a personal computer.

Histological Analysis. Tumor-bearing animals were sacrificed by administration of an overdose of i.p. anesthesia, followed by cervical dislocation. The tumors were then dissected along with the skin and s.c. tissues and placed in 10% buffered formalin. Tissue sections were obtained at 5-μm thickness and stained with H&E. Mitotic counts were obtained by morphological evaluation of 20 high-power (×40) fields for each animal. Areas of gross cell death were quantified using image morphometry, whereby area of tumor death was divided by the total tumor area as measured by ImageTool software (developed at the University of Texas Health Science Center, San Antonio, TX).

Statistical Analysis. Tumor glycolytic and lactate clearance rate constants as well as Eppendorf pO$_{2}$ values are reported as mean ± SE. The mean pO$_{2}$ value from treated and control tumors was obtained by first averaging the pO$_{2}$ values from each individual tumor and then averaging these values across the two groups of animals. The Mann-Whitney rank-sum test was used to compare control and treated animals. $P < 0.05$ (95% confidence level) was considered significant.

RESULTS

Inhibition of Tumor Growth in Vivo by Cp. Tumor volumes for control and Cp-treated tumors as a function of time after tumor cell inoculation are shown in Fig. 1. A significant growth delay was observed 2 days after treatment with Cp ($P < 0.05$). Control tumors reached a volume of 1000 mm$^{3}$ in 19 days, whereas treated tumors reached the same volume in 25 days, thereby exhibiting a growth delay of more than 6 days.

Tumor Blood Flow by Doppler Ultrasound. The average preinfusion blood flow velocity was determined to be 8.8 cm/s. There was no change in the flow velocity during glucose infusion (9.0 cm/s) or at the end of glucose infusion (8.3 cm/s).

Cp Treatment Causes a Drop in Glycolytic rate of RIF-1 Tumors. Fig. 2 shows a representative set of the lactate and glucose regions of baseline subtracted $^{13}$C spectra. $[^{1}$-$^{13}$C$]$glucose resonances were observed at 92 and 96 ppm within 5–10 min after the start of glucose infusion in all tumors. The peak area of glucose increased steadily for 25–30 min, reached a plateau, and started to decrease after the glucose infusion was stopped (40 min). The lactate resonance appeared after 20–25 min of infusion, continued to increase, and stayed at a steady level until the end of experiment (100 min; Fig. 2). In vivo $^{1}$C labeling of other metabolites was not observed. Glycolytic rate constants calculated by the two-compartment model (21) before Cp or saline treatment ranged between 0.027 and 0.141 min$^{-1}$. Due to
this inherent intratumor variability, normalized changes (i.e., percentage change) in tumor glycolytic rate constants, rather than absolute rate constants, have been shown in Fig. 3. A >50% decrease in glycolytic rate constant was observed 24 h after treatment with Cp ($P < 0.01$). Control tumors did not show any significant difference in the glycolytic rates 24 h after saline treatment ($P = 0.49$). Analysis of individual tumors indicated a consistent decrease in glycolytic rate for all treated tumors compared with pretreatment values. Control tumors did not exhibit this decrease in glycolytic rate constants (data not shown). Lactate clearance rate constants ranged between $0.001$ and $0.075$ min$^{-1}$. We did not observe any significant change in these rates in either the treated ($P = 0.44$) or control tumors ($P = 0.83$).

**Increased [3-13C]Glutamate and [4-13C]Glutamate Labeling in Cp-Treated Tumors Observed by 13C MRS of Tumor Extracts and by HPLC.** Representative high-resolution 13C spectra of a control tumor (bottom trace) and a treated tumor (top trace) are shown in Fig. 4. The labeling of [3-13C]lactate was substantially higher than [4-13C]glutamate in both treated and control tumors; however, the [4-13C]glutamate to [3-13C]lactate ratio in treated tumors (0.68; $n = 4$) was almost double compared with that of the control tumors (0.35; $n = 3$). Similarly, the [C-3]/[C-4] glutamate ratio from treated tumors (0.57) was higher than that from untreated control tumors (0.33). The increase in the glutamate pool in treated tumors was also observed by HPLC. The total glutamate concentration in the treated tumors was $2.54 \pm 0.3$ mmol/g wet weight of tumor compared with only $1.45 \pm 0.2$ mmol/g wet weight in the control tumors.

**RIF-1 Tumors Exhibit a Decrease in $pO_2$ Values after Cp Treatment.** Histograms of the pooled $pO_2$ distribution for all tumors in the two groups are shown in Fig. 5. Treated tumors showed a left shift in the frequency distribution, indicating a substantial decrease in the $pO_2$ of the treated tumor. The average $pO_2$ value for treated tumors (7.8 $\pm$ 0.5 mm Hg) was significantly lower than that of the control tumors (13.2 $\pm$ 1.7 mm Hg; $P < 0.05$). In two control and treated tumors, $pO_2$ values were measured before and 24 h after treatment with Cp. A marked decrease in $pO_2$ values of treated tumors was observed in these tumors, whereas no changes in tumor $pO_2$ values were observed in control tumors (data not shown).

**Increased PN Levels after Cp Treatment.** The spatial distributions of PN (NADH) levels through various sections from a representative control (Fig. 6A) and Cp-treated (Fig. 6B) tumor are shown. Increased PN fluorescence, which reflects an increase in the NADH concentration, is clearly observed throughout the Cp-treated tumor. The reduced form of PN (NADH) emits fluorescence when excited with UV light (31). PN fluorescence reflects the concentration of reduced electron carrier entering the electron transport chain.

**Cp-Induced Growth Arrest Correlates with Decreased Mitosis.** The histology of the untreated RIF-1 tumor was characterized by solid sheets of spindle and epithelioid cells and a high mitotic activity (Fig. 7A). Treated tumors showed a marked decrease in mitotic counts (Fig. 7B), similar to that reported earlier with 200 mg/kg Cp treatment (32). Areas of gross necrosis or cell death were not observed in either the control or Cp-treated tumors 24 h after treatment.

**DISCUSSION**

In this study, it was shown that 24-h treatment with Cp caused a significant drop in the glycolytic rates of RIF-1 tumors as measured by $^{13}$C MRS in vivo. The decrease in glycolysis was accompanied by an increase in [C-4] glutamate labeling, an increase in the [C-3]/[C-4] glutamate labeling, a significant decrease in tissue $pO_2$, and an increase in NADH levels. These data indicate that the decrease in glycolysis is due to increased oxygen consumption and a shift toward oxidative phosphorylation of the Cp-treated tumors. Moreover, the observation that tumor energy metabolism shifts after treatment with cytotoxic therapy may indicate metabolic sites for secondary or adjunct interventions.

**Tumor Glycolytic Rates by $^{13}$C MRS.** We have reported previously a decrease in steady-state lactate level of Cp-treated tumors (8).
This is consistent with a mechanism in which reduced lactate is observed due to a diminution in lactate production by the fewer surviving cells after therapy. Earlier 31P NMR studies had paradoxically shown an increase in ATP/Pi ratio in Cp-treated RIF-1 tumors, suggesting an increased bioenergetic status (33). Increased cell kill and clearance would cause a decrease in interstitial pressure, leading to increased oxygen availability and thus a shift toward oxidative phosphorylation of the surviving tumor cells that could account for the observed increase in the ATP/Pi ratio. Increased perfusion and thus increased oxygen availability have, in fact, been reported for Cp-treated RIF-1 tumors (34). Our observation of increased [C-4] glutamate labeling along with an increase in the [C-3]/[C-4] glutamate ratio and an increase in the glutamate pool size in Cp-treated tumors is consistent with an increase in the TCA cycle activity (35) along with fractional enrichment of [C-4] glutamate of the surviving tumor.

The hypothesis that a decrease in tumor interstitial pressure would lead to increased oxidative metabolism is supported by the observed decrease in tissue pO2 in Cp-treated tumors relative to untreated controls, which is consistent with increased oxygen consumption by surviving cells. A recent study by Nielsen et al. (19) reported that carbogen (95% O2, 5% CO2) breathing led to an increase in tumor pO2 (measured by Eppendorf electrodes) in the highly hypoxic C3H murine mammary carcinomas. The increase in pO2 was accompanied by a decrease in glycolytic rate and an increase in [C-4] glutamate labeling. Although these results indicate increased TCA cycle activity, the saturation of blood and tissue oxygen levels by carbogen breathing may have masked any increased oxygen consumption measured by pO2 levels in their study. Alternatively, differences in the change of pO2 values of these two tumor types might be due to the differences in the steady-state hypoxic status of these tumors. Whereas the C3H

**Fig. 2.** Sequential baseline corrected 1H-decoupled and nuclear Overhauser effect-enhanced 13C magnetic resonance spectra of an in vivo RIF-1 tumor showing the glucose and lactate resonances. The total acquisition time for each spectrum was approximately 5 min using a repetition time of 1 s and 256 acquisitions. The two-headed arrow indicates the time of glucose infusion (40 min).

**Fig. 3.** Normalized glycolytic rates (percentage changes) from cyclophosphamide-treated (filled bars; n = 8) and control tumors (dashed bars; n = 6) showing a significant decrease in glycolytic rate constants 24 h after Cp treatment (* indicates P < 0.01), whereas no significant change after sham treatment was observed.
tumor line is very hypoxic, the RIF-1 tumor has a very low hypoxic fraction, as suggested by the higher pO$_2$ values.

The interpretation of a shift toward oxidative phosphorylation after Cp treatment is further supported by the observation that NADH levels increased, indicating a reduced state of the more hypoxic treated tumors. In the case of increased oxygen delivery (by increased perfusion), one would have expected to see an increase in the NAD$^+$ with a concurrent drop in the NADH levels. However, a slight shift in tumor metabolism from glycolysis toward oxidative phosphorylation would lead to a rapid increase in the ATP mass action ratio, consistent with the previous observation of increased ATP/Pi ratio (33). As this ratio increases, the rate of electron transport decreases, leading to an increase in NADH levels (36) and feedback inhibition of the enzymes in the citric acid cycle and in the glycolytic pathway that have NAD$^+$ cofactors (36). The increase in ATP caused by a shift toward oxidative metabolism from anaerobic glycolysis would also lead to the decrease in phosphofructokinase, which is the rate-limiting enzyme in glycolysis.

It should be noted that the increased ATP levels and decreased glycolytic rates of treated tumors could also arise if the tumor shuttles the accumulated [C-3] lactate (due to the infusion protocol) into the bloodstream; [C-3] lactate then gets converted into glucose in the liver via the Cori cycle (37). Alternatively, under sufficient oxygen delivery, accumulated extracellular lactate can return to the intracellular space through the reversible monocarboxylate carrier and be oxidized (38). However, we think that this possibility is unlikely because (a) blood glucose concentration was kept constant at 17 mM during the infusion protocol, making it unlikely that the tumor would preferentially use lactate over glucose; (b) lactate oxidation would probably be detected by increased labeling in other metabolites; and (c) the Cori cycle usually occurs in the liver in cachectic patients, and no weight loss was observed in these animals over the experimental period.
Effects of Hyperglycemia. Labeling of glucose with $^{13}$C provides a unique noninvasive method of measuring glycolytic metabolism of tumors in vivo using $^{13}$C MRS (4, 9, 14, 16, 17, 19–21, 39–42). Infusion of [1-$^{13}$C]glucose is generally maintained for extended periods of time to observe the conversion of glucose to lactate (9, 19–21). The plasma glucose levels, due to this infusion protocol, exceed physiological ranges and induce acute hyperglycemia. Increased blood glucose levels may also alter tumor blood flow (43) and reduce oxygen consumption (44) of some tumors. This phenomenon was first observed by Crabtree (45) and is known as the “Crabtree” effect. We have previously reported an absence of the Crabtree effect in this tumor model using the same infusion protocol as used in this study (24), and thus we believe that the effects of hyperglycemia on tumor blood flow and oxygen consumption in this study were negligible. Minimal changes in intra- and extracellular pH during glucose infusion suggested that tumor blood flow was also not altered significantly (24). Other studies have shown that sustained elevated hyperglycemia did not alter tumor pH in human melanoma xenografts (46). However, Traykov and Jain (47) have shown that the RBC membrane elastic modulus increases with hyperglycemia, which could lead to alteration of blood flow independent of changes in pH. In the present study, glucose was infused i.v. to achieve a steady-state blood glucose

![Fig. 6. Fluorescence imaging of RIF-1 tumors exhibiting the pyridine nucleotides (NADH) levels from a control tumor (A) and a cyclophosphamide-treated tumor (B). The color bar indicates relative levels of NADH on an arbitrary scale from 0 to 255. Increased NADH levels are evident in all of the slices from the treated tumor compared with the control.](image)

![Control](image)

![Cp](image)

Fig. 6. Fluorescence imaging of RIF-1 tumors exhibiting the pyridine nucleotides (NADH) levels from a control tumor (A) and a cyclophosphamide-treated tumor (B). The color bar indicates relative levels of NADH on an arbitrary scale from 0 to 255. Increased NADH levels are evident in all of the slices from the treated tumor compared with the control.

Fig. 7. H&E-stained sections at ×40 magnification from RIF-1 tumors. The left panel shows a section from a control tumor, whereas the right panel is from a cyclophosphamide-treated tumor. The left panel shows two mitotic cells (open arrowheads). The mitotic indices for the control tumors were 8/high-powered field, which was reduced to 2/high-powered field after treatment.
level of 17 mm, and no significant change in tumor blood flow velocity was observed with this protocol using the Doppler ultrasound method. Furthermore, because we did not observe any significant changes in the glycolytic rate constants of sham-treated controls, we believe that our results are due to a change in tumor metabolism and not due to an artifact caused by the infusion protocol.

The kinetic model used in this study to calculate glycolytic and lactate clearance rates was initially proposed by Artemov et al. (21). Several assumptions are made in this model, which are generally applicable to many tumors. First, tumor metabolism is assumed to be predominantly glycolytic. This assumption holds true in our study because the [C-3] lactate to [C-4] glutamate ratio in untreated tumors was 28:1, suggesting that glycolysis is the predominant mechanism of glucose consumption. Although this ratio decreased to 14:1 after treatment with Cp, the validity of the above assumption still holds. Second, the model assumes that the [13C]glucose signal observed in vivo is predominantly from the extracellular and interstitial compartments. Artemov et al. (21) have previously shown that the intracellular levels of [13C]glucose are negligible in RIF-1 tumors, the tumor model used in this study. Third, the model assumes that the tumor is homogeneous with negligible necrotic fraction. Histological studies of both the control and Cp-treated tumors did not indicate any gross necrotic areas with a decrease in mitotic index of the treated tumors, indicating that the tumors were relatively homogeneous.

The model proposed by Artemov et al. (21) also provides an estimate of the lactate clearance rate constant from the interstitium into the vasculature. Because Cp treatment causes increases in both tumor gadolinium-diethylenetriaminepentaacetic acid uptake (8) and tumor perfusion (34) in RIF-1 tumors, we expected an increase in lactate clearance rate constants after therapy. However, no significant differences in lactate clearance rates of Cp-treated tumors were observed. It is not clear why such changes were not observed in our study, but a similar observation was reported in C3H murine mammary carcinomas after carbon monoxide breathing (19). In this study, the lack of change in lactate clearance rates was hypothesized to be due to the opposing effects of a decrease in tumor blood flow (caused by carbon monoxide) along with the effect of a positive intracellular to extracellular pH gradient on tumor lactate levels. This, however, is not the case in RIF-1 tumors, where a nonsignificant change in pH has been reported after 24 h of Cp treatment (33).

The current study points toward the importance of a multiparametric study for a better understanding of the metabolic and biochemical changes in a tumor after therapeutic interventions. It is clear from our study that a combination of different techniques (13C MRS, Eppendorf electrodes, and fluorescence imaging) helps in proper interpretation of the biochemical changes in tumors. Had these complementary measurements not been performed, one might have been led to conclude that Cp treatment causes hypoxia in RIF-1 tumors resulting from decreased oxygen availability rather than increased oxygen consumption and a shift toward oxidative phosphorylation.

It should be noted that our studies have been performed on a single tumor type that, unlike many other tumor models, has a low hypoxic fraction. Thus, the general applicability of these results to other tumor types with higher hypoxic fraction needs to be tested. These changes in glycolytic rates might also depend on the mechanism of action of the therapeutic modality. For example, if an antivasular agent is used, the glycolytic rates would probably increase because the delivery of oxygen to the tumor cells would be substantially reduced, making them more glycolytic.

In summary, treatment of RIF-1 tumors with Cp leads to a significant decrease in glycolytic rates 24 h after treatment, at which time no significant difference in the tumor volume was detected between Cp-treated and control tumors. These studies suggest a role of 13C MRS in monitoring early treatment response to tumors and provide an understanding of tumor metabolism after chemotherapy. Additional studies to enhance the sensitivity of 13C MRS using 1H-13C cross-polarization techniques (14) and inverse detection of 1H detection of scalar 13C-1H coupling (20) along with [1,6-13C]glucose infusion might assist in detection of glutamate/glutamine labeling in vivo. These studies would facilitate measurement of TCA cycle activity using elaborate kinetic models developed by Chance et al. (48) or Chatham et al. (49).

ACKNOWLEDGMENTS

We sincerely thank Dr. Dimitri Artemov (Johns Hopkins University) for providing the 13C data analysis program, Dr. Rolf Gruter (Minnesota) for providing the bi-level nuclear Overhauser effect pulse sequence, Dr. Suzanne Wehrli (Children’s Hospital of Philadelphia) for help in performing the high-resolution extract studies, Dr. Michael B. Robinson (Children’s Hospital of Philadelphia) for assistance with HPLC analysis, and Dr. Chandra M. Sehgal (Department of Radiology, University of Pennsylvania) for help in measuring blood flow velocities by Doppler ultrasonography. Dr. Robinson is a recipient of NIH Grant P30-HD26979.

REFERENCES


Cyclophosphamide Treatment Modifies Tumor Oxygenation and Glycolytic Rates of RIF-1 Tumors: 13C Magnetic Resonance Spectroscopy, Eppendorf Electrode, and Redox Scanning


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/24/8813

Cited articles
This article cites 41 articles, 9 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/24/8813.full.html#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
/content/63/24/8813.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.