Effects of hyperglycemia on oxygenation, radiosensitivity and bioenergetic status of subcutaneous RIF-1 tumors

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Abstract. Since tissue oxygen tension is a balance between delivery and consumption of oxygen, considerable effort has been directed at increasing the former and/or decreasing the latter. Techniques to decrease the rate of cellular oxygen consumption (increasing the distance oxygen can diffuse into tissues) include increasing glycolysis by administering supra-physiologic levels of glucose. We have examined the effect of hyperglycemia produced by intravenous glucose infusion on the tissue oxygenation and radiation response of subcutaneously implanted murine radiation induced fibrosarcomas (RIF-1). A 0.3 M glucose solution was delivered via tail vein injection according to a protocol that maintained glucose at a plasma concentration of 17±1 mM. The effect of this treatment on radiation response (clonogenic and growth delay studies), tumor oxygenation (needle electrode pO2 and 2-[2-nitro-1H-imidazol-1-yl]-N-(2,2,3,3,3-pentafluoropropyl) acetamide (EF5) binding), and tumor bioenergetics and pH (³¹P NMR spectroscopy) was examined. Systemic measurements included hematocrit and blood glucose and lactate concentrations. The results of these studies suggest that these subcutaneously implanted RIF-1 tumors are both radiobiologically and metabolically hypoxic and that intravenous glucose infusion is not an effective method of modifying this metabolic state.

Introduction

Tissue oxygen tension is an important determinant of radiosensitivity. Since tissue oxygen tension is a balance between delivery and consumption of oxygen, considerable effort has been directed at increasing the former and/or decreasing the latter. Techniques to increase oxygen delivery include hyperbaric oxygen (1) and carbogen breathing (2). To decrease the rate of cellular oxygen consumption (and consequently increase the distance oxygen can diffuse into tissues), efforts have been directed at inhibiting the mitochondrial respiratory chain (3-5), or by increasing glycolytic flux (6) through administration of supra-physiologic levels of glucose. Crabtree first noted that hyperglycemia produces a decrease in oxygen consumption in some tumors (7). Since induction of a Crabtree effect should enhance tumor radiosensitivity, its effect on radiation response has been studied earlier. In two reports (6,8), glucose administration did not improve radiation response. In other studies, there were disparities in the effects of systemic hyperglycemia on tumor blood flow, pH and bioenergetic status (9-11). These disparities may be related to the use of different tumor cell lines/animal models, routes of glucose administration, and techniques for measuring tumor pH and blood flow. The current investigation was undertaken to evaluate the effect of hyperglycemia produced by intravenous glucose infusion on the radiation response of subcutaneously implanted radiation induced fibrosarcomas (RIF-1). A modification of the previously reported intravenous glucose infusion protocol developed for ¹³C MRS of murine tumors was employed (12). This protocol rapidly increases and maintains the blood glucose concentration at 17±1 mM. The effect of this treatment on tumor oxygenation was measured using multiple independent endpoints as well as radiation response. Radiation response was determined by clonogenic assay and growth delay. Tumor oxygenation was monitored by needle electrode (Eppendorf) pO2 measurements and 2-nitroimidazole (EF5) binding. Tumor bioenergetics and pH were determined by ³¹P NMR spectroscopy. Systemic measurements of hematocrit as well as glucose and lactate concentrations were performed. These investigations demonstrated that contrary to a number of previous reports, (13-17) the subcutaneously implanted RIF-1 tumors that we and at least one other laboratory (18) have examined are hypoxic, and intravenous glucose infusion is not an effective means for modifying this metabolic state.

Materials and methods

Tissue culture. RIF-1 tumor cells were obtained from Dr Barbara Henderson, Roswell Park Cancer Institute, Buffalo, NY and were grown in monolayers using RPMI 1640 culture medium containing 10% fetal calf serum, 1% penicillin/streptomycin and 0.15% L-glutamine (‘medium’). They were
performed by irradiating RIF-1 cells in cell culture dishes
on an orthovoltage X-ray unit operated at 225 kVp and 13-mA,
infusion protocol. Blood glucose and lactate levels were
maintained at 17 ± 1 mM. Saline was infused into control animals using the same
infusion protocol (data not shown). The hypoxia survival curve was
determined by administering graded doses of radiation to
pre-determined tumor volume of 1500 mm$^3$ for control vs.
pre-determined tumor volume of 1500 mm$^3$ for control vs.
control tumors examined by $^{31}$P NMR spectroscopy.

Animals and tumors. All animal studies were performed in
accordance with the regulations of the University of
Pennsylvania Institutional Animal Care and Use Committee.
Female, 18-20 g C3H mice (Taconic Farms Inc., Germantown,
NY, USA), approximately 6 weeks of age, were used. Tumors
were propagated by inoculating 3x10$^6$ cells, in a 30 µl volume,
subcutaneously in the upper thigh. Tumor volumes were
measured three times weekly in three dimensions, and tumor
volume was calculated from the formula $V = \pi (x \times y \times z)/6$,
where x, y, and z are three perpendicular diameters of the
animal.

Animals were anesthetized with an intraperitoneal injection
of 153 mg/kg ketamine (Fort Dodge Animal Health, Inc., Fort
Dodge, IA, USA) and 1.53 mg per kg acepromazine (Fermenta
Animal Health Co., Kansas City, MO, USA). A 26G Abbocath-
T catheter (Abbott Ireland, Sligo, Republic of Ireland) was
placed in the tail vein for glucose/saline infusion. Glucose
(0.3 M) was infused at a controlled rate to rapidly raise and
maintain the steady state blood glucose concentration at 17±1
mM. Saline was infused into control animals using the same
infusion protocol. Blood glucose and lactate levels were
measured by drawing blood from the digits every 15 min.
Hematocrit was measured following the infusion protocol by
taking blood from the digit directly into a microhematocrit
tube.

Radiation studies
Irradiation procedures. Irradiation studies were performed
on an orthovoltage X-ray unit operated at 225 kVp and 13-mA,
0.2 mm copper filter. The dose rate was 3.4 Gy per minute at
a source to tumor distance of 30 cm. In vitro experiments were
performed by irradiating RIF-1 cells in cell culture dishes
with doses of 0-12 Gy. In vivo studies using irradiation doses
of 0-20 Gy were performed 12 days after tumor implantation.
At this time, tumors ranged from 200-400 mm$^3$ in volume.
Relatively large tumors were utilized so that comparisons could
be made with tumors examined by $^{31}$P NMR spectroscopy.

Radiation response studies. RIF-1 cells for in vitro irradiation
studies were removed from tissue culture flasks during the
logarithmic growth phase by administration of 4 ml 0.05%
Trypsin-EDTA for 5 min at room temperature. The trypsin
activity was blocked by adding 6 ml of fetal calf serum-
containing medium.

For determination of hypoxic cell response, cells dissociated
from tumors were irradiated under hypoxic conditions as
previously described (19). The oxic survival curve was
generated by administering graded doses of radiation to
aerated cells dissociated from RIF-1 tumors. One of two
different glucose concentrations (11 or 22 mM) was used in
the medium, and no difference in the radiation response was
detected (data not shown). The hypoxia survival curve was
generated by irradiation of six tumors in euthanized mice
(e.g. in vivo conditions; see below) and by irradiation under
hypoxic conditions of single cell dispersions of two tumors
(e.g. ex vivo conditions).

In vivo and in vitro studies. The data points for the various
experimental conditions were generated by irradiation of air
breathing animals while they were receiving the appropriate
intravenous infusions (e.g. glucose or saline). For hypoxic
tumor irradiation, animals were euthanized 10 min before
irradiation. Tumor dissociation proceeded as previously
described (20). Briefly, immediately after the irradiation
tumors were excised, minced, suspended and stirred in 10 ml
of an enzyme cocktail (0.075% pronase, 0.03% collagenase,
0.03% DNAses) for 30 min at 37°C. Enzyme digestion was
stopped by addition of 10 ml of cold fetal calf serum-containing
medium, and the suspension was filtered through a 70 µm
strainer. The cells were counted (Coulter Counter, Coulter
Corporation, Miami, FL), centrifuged and the pellet was
resuspended in culture medium at 10$^6$ cells/ml. The viability
of the cells was determined, and dye excluding cells were
counted with a hemocytometer. Suitable numbers of cells
were plated in 100 mm dishes, containing 9 ml medium. The
number of cells plated was varied to yield 100-200 colonies/
plate. In this range, the number of colonies varies linearly
with the numbers of RIF-1 cells seeded. Multiple replicates
were plated for each dilution. The plates were incubated for
10-12 days followed by staining and counting of colonies.

The data from the survival studies was fit to the equation:

$$S = S_o e^{D - \beta D}$$

where D, radiation dose; S, survival fraction and $S_o$, survival
fraction at 0 Gy irradiation. A quadratic fit was made to
determine the best-fit coefficients.

Growth delay studies. Tumors were irradiated 12 days after
implantation. The difference in the time required to reach a
pre-determined tumor volume of 1500 mm$^3$ for control vs.
test groups defined the growth delay.

Glucose infusion protocol and assay. A 0.3 M glucose solution
was infused into the tail vein using a Harvard ‘33’ double
syringe pump (Harvard Apparatus, Inc., Holliston, MA, USA).
The infusion rate was adjusted to obtain a steady state blood
glucose level of 17±1 mM within 10 min of infusion. The
infusion rate was then dropped in steps to maintain a steady
blood glucose level of 17±1 mM for the remaining 30 min of
infusion. Various concentrations of glucose in the infusate
and rates of infusion were tested on matched cohorts of mice
to determine the optimum infusion protocol (data not shown).
Blood glucose and lactate levels were assayed with a Glucose-
Lactate Analyzer (YSI 2300 STAT plus; YSI Inc., Yellow
Springs, OH, USA).

Measurements of tumor tissue oxygenation
Needle electrode studies. Tumor pO$^2$ measurements were
performed on anesthetized mice with an O$^2$ sensitive needle
electrode (KIMOC 6650, Sigma pO$^2$ Histogram; Eppendorf,
Hamburg, Germany). The protocol for such measurements
has been previously described (21). 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 in order to relieve tissue pressure. Local oxygen
pressures were measured one second after the backward
motion. Since these tumors were to be irradiated and re-measured with the Eppendorf electrode, only a single track was made, generating between 20-30 pO2 values. Measurements were performed immediately after the animal was anesthetized, prior to and 40 min after infusion and/or irradiation. Negative values of pO2 were set to zero for all the experiments (22).

EF5 studies. Mice were given 2-[2-nitro-1H-imidazol-1-yl]-N-(2,2,3,3,3-pentafluoropropyl) acetamide (EF5) as an intravenous injection of 10 mM drug prepared in 0.9% saline for detection of the presence of hypoxia, as described previously (23). The volume of solution administered was 1% of the animal’s weight, and the resulting equivalent whole-body concentration was 100 µM. Three hours following EF5 administration, the animal was anesthetized and radiation administered (see above); the tumor was rapidly excised, weighed, cooled by placing tissues on an iced surface, and bisected. Portions of the tumor were desegregated for plating efficiency studies (see below) and analysis of EF5 binding by flow cytometry.

The staining of cells for analysis of EF5 binding and flow cytometry was performed as described earlier (24). Briefly, cells were fixed in 4% paraformaldehyde for 1 h, rinsed three times in phosphate buffered saline (PBS) and blocked overnight. After removing the blocking solution, cells were stained for 4-6 h in 75 µg/ml ELK3-51: Cy5 and rinsed twice in PBS containing 0.3% Tween 20. The final rinse was in PBS, and cells were stored in 1% paraformaldehyde until analyzed. Appropriate control studies were performed as previously reported (25), that confirmed the absence of background fluorescence and non-specific binding (data not shown). All procedures and storage were performed at 0-4°C.

Flow cytometric analyses was performed with a FACS Calibur (Becton Dickinson, San Jose, CA) maintained by the Flow Cytometry Facility at the University of Pennsylvania Cancer Center. Cells to be analyzed were suspended at 0.5-1x106 cells/ml in 1% paraformaldehyde and filtered to eliminate aggregates. Preceding each session, machine settings were adjusted until a cellular standard produced the same absolute fluorescence. This standard was created by treating V79 cells in vitro with 100 µM EF5 at <0.005% O2 for 4 h. The standard was stained for EF5 in parallel with the experimental samples, and its Cy5 fluorescence was set to 1000. All samples were read on the FL4 channel (excitation 635 nm and emission 661 nm) with a threshold forward scatter of 30. Data were plotted as histograms (cell count vs. fluorescent intensity) in Cell Quest (Becton Dickinson, Mountain View, CA) and read into Wingz (Investment Intelligence Systems Corporation, Lenexa, KS) using FCS Assistant v1.3a beta (shareware, www.bio.umass.edu). Cumulative frequency data were calculated in Wingz.

NMR spectroscopy. 31P spectra were acquired from anesthetized tumor-bearing mice with a 9.4T, vertical bore Omega CSI spectrometer (GE, Medical System, Fremont, CA) using a double tuned surface coil resonating at 161.25 MHz for 31P and 105.5 MHz for 23Na. Experimental parameters were: sweep width, 15 K; recycle delay time, 2 sec; pulse width, 15 µs; data points, 8 K and number of scans, 256. A 1 cm single loop surface coil was constructed to fit around the tumor (tumor volume was between 200-600 mm3). As an external reference, a small glass bulb containing 80 mM dimethylphosphonate (DMMP) was placed on top of the tumor in the center of the coil. An extracellular pH marker (pH2: 3-aminoaliphatic phosphonate, 3-APP) was administered by intraperitoneal injection (1.1 g/kg). Core body temperature was maintained at 37°C by placing the mouse on a water-heated pad. The B0 field was shimmed on the 23Na signal of the tumor prior to data acquisition (typical linewidth, 50-80 Hz). Post-processing included application of 30 Hz line broadening. Spectral peak areas were estimated by fitting the spectra using Nuts Software (Acorn NMR, CA, USA).

Intracellular tumor pH (pHi) was estimated from the chemical shift of inorganic phosphate (P) relative to PCr (at -2.54 ppm) using equation i). The extracellular pH (pHe) was estimated from the chemical shift of 3-APP relative to phosphocreatine (PCr) using equation ii) (26) where δ is the chemical shift:

\[
\begin{align*}
\text{pHi} &= 7.169 + \log \left[ \frac{(\delta-0.77)/(3.16-\delta)}{(\delta-2.03)/(23.84-\delta)} \right] \quad \text{ii) } \\
\text{pHe} &= 7.11 - \log \left[ \frac{(\delta+0.77)/(3.16+\delta)}{(\delta+2.03)/(23.84+\delta)} \right] \quad \text{ii)}
\end{align*}
\]

Resonance assignments were made based on the 31P NMR studies of RIF-1 tumor extracts by Evanochko et al (27).

Statistical analysis. Unpaired Student’s t-test was applied for statistical evaluations; all measured values are shown as mean ± SEM of each group. To analyze the oxygen status in tumors, pO2 values from tumors of the same experimental group were pooled, and a Mann-Whitney rank sum test was performed.

Results

Effects of glucose or saline infusions. Fig. 1 displays the blood glucose levels measured in mice during the 40-min infusion period. In control animals, subjected to saline infusion, the glucose level remained constant at 5.8±0.2 mM. Animals in which 0.3 M glucose was infused, the blood glucose level rose to 17.8±0.7 mM within 10 min and was maintained at this level for 40 min. Blood lactate levels were constant at
1.5±0.1 mM and 2.1±0.1 mM during the 40-min study period for saline and glucose infusions, respectively.

The baseline hematocrit percentage was 37.0±0.3. The hematocrit percentage decreased to 33.7±1.0 and 33.5±0.6 after saline or glucose infusion, respectively, and was significantly different from the baseline values (p<0.05).

**Radiation response studies.** Tumor volumes, prior to irradiation, were 434±34 mm$^3$ for untreated controls (n=4); 377±37 mm$^3$ for tumors irradiated but without any infusion (n=2); 441±83 mm$^3$ for mice infused with saline (n=4) and 409±56 mm$^3$ for mice infused with glucose (n=5). The tumor volumes in these 4 groups were not significantly different (p>0.05). Tumor volumes of mice irradiated for *in vivo* and *in vitro* plating efficiency studies were 213±12 mm$^3$. While this volume is significantly lower than those of tumors studied for growth delay (p<0.05), subsequent measurements indicated that these differences did not affect measurements of tumor oxygenation (see below).

The plating efficiency (0 Gy) of tumors excised from air breathing mice was similar to that obtained from tumors obtained from euthanized (hypoxic) animals (0.38±0.008 and 0.352±0.060, respectively). Tumor cell survival following 20 Gy irradiation of air breathing mice, was 0.068±0.020 and 0.048±0.003 for mice infused with glucose and saline, respectively. The data obtained under these two conditions were not statistically different (p>0.05) (Fig. 2A). The radiation response of tumor cells from air breathing animals was not statistically different from those of hypoxic tumor cells or animals, suggesting that these RIF-1 tumors were hypoxic in air breathing mice. The oxygen enhancement ratio for cells from these tumors (ratio of slopes of linear portions of semi-logarithmic cell survival curves for anoxic vs. aerobic cells, respectively) was 2.96, which is close to the value of 3 that is, generally attributed to the oxygen effect (28). It is to be noted that the survival curve for air breathing animals did not differ significantly from that of the anoxic cells.

Growth delay studies demonstrated that untreated control tumors (n=4) reached a volume of 1500 mm$^3$ in 6 days from a volume of 250 mm$^3$ (Fig. 2B). For irradiated tumors, an average of 10 additional days was required to reach 1500 mm$^3$ irrespective of the infusion protocol (control, saline infusion, and glucose infusion). Because of the small number of animals employed in these studies, significance levels have not been determined; however, there is no trend of radio sensitization by glucose infusion. These studies indicate that hyperglycemia had no significant effect on tumor radiosensitivity.

**Eppendorf needle electrode measurements** (Fig. 3). The median tumor pO$_2$ value (n=6) was 3.8 mm Hg before and 1.9 mm Hg (p<0.001) after saline infusion. The median pO$_2$ values before glucose infusion (n=5) was 2.7 mm Hg, which decreased to 1.8 mm Hg (p<0.001) after 40 min of glucose infusion. Irrespective of the agent infused (glucose vs. saline), a statistically significant decrease in median pO$_2$ was seen (p<0.001).

**EF5 flow cytometry studies.** Twelve tumors (204-300 mm$^3$) were used to determine baseline levels of hypoxia-related EF5 binding. Cells desegregated from tumors generally exhibited similar EF5 binding curves indicating a small fraction of severely hypoxic cells (Fig. 4A) (e.g. cells with a fluorescence >100). The typical pattern of fluorescence from these RIF-1 cells is shown as open diamonds. The figure also illustrates the EF5 binding distribution in two tumors with atypical binding (open squares and triangles). One tumor (open squares) has almost no EF5 binding indicating that it was oxic, and another tumor (open triangles) had intermediate levels of EF5 binding. Absolute levels of binding can range from 10-1000 absolute fluorescence based on cellular standards.
using techniques previously described for V79 cells (25). Fig. 4A demonstrates EF5 binding in a RIF-1 tumor that exhibits minimal binding with cellular fluorescence greater than 100 (open squares) in comparison to another RIF-1 tumor (open diamonds) that exhibits substantial high binding, including cellular fluorescence up to 1000. The presence of higher levels of EF5 bindings indicates more cell-bound drug and lower oxygen tensions. The data from these tumors are also summarized as cumulative frequency plots of EF5 distributions (Fig. 4B). From this, both intra- and inter-tumoral heterogeneity of binding can be seen. The median absolute fluorescence for these tumors was between 7 and 65 and the 75% value was between 14 and 140. Based upon calibration studies performed on RIF-1 tumors (25), the latter values suggest the presence of hypoxia with a substantial proportion of the cells at pO2 values between one and 7 mm Hg. A correlation between tumor size and median EF5 binding was not observed (data not shown). A general trend between lower Eppendorf values and higher binding was detected, but it was not statistically significant (data not shown).

Figure 3. Distribution of pO2 values in RIF-1 tumors, based upon Eppendorf electrode values before and after saline or glucose infusions.

Figure 4. (A), Histograms derived from flow cytometric analysis of EF5 binding in cells from RIF-1 tumors. The flow cytometer was adjusted to provide signal intensity with a median signal of 1000 for WNRE (25) calibration cells (closed circles). Most of the twelve tumors studied had binding patterns similar to the open diamonds, two outliers were noted: one tumor had intermediate but not maximally bright binding (open triangles) and one tumor had no hypoxic cells (open squares). (B), Cumulative frequency analysis of the same tumors illustrated in (A). The majority of tumors (open diamonds) had about 25% cells with binding levels between 100 and 1000. Based on in vitro studies, such cells would be expected to have oxygen levels less than 1% (7.5 mm Hg).
NMR spectroscopy. The $^{31}$P NMR spectrum of a subcutaneous RIF-1 tumor is displayed in Fig. 5. Most of the resonance assignments are indicated in the figure. Abbreviations are as follows: α-, β- and γ-phosphates of nucleoside triphosphates (NTP), phosphocreatine (PCr), inorganic phosphate (Pi), phosphate monoesters (PME), 3-aminopropylphosphonate (3-APP) and dimethylmethylphosphonate (DMMP).

Collectively, these peaks are referred to as the phosphodiester (PDE) peak(s). The Pi peak at 1.53 ppm is an indicator of pH since its chemical shift measures the equilibrium levels of H$_2$PO$_4^-$ and HPO$_4^{2-}$. The phosphomonoester peak (PME) peak is observed at 4.25 ppm and originates primarily from phosphoethanolamine (PE, at ~4.3 ppm) and phosphocholine (PC at ~4.0 ppm). The PMEs are precursors of phospholipids, whereas the PDEs are products of phospholipid catabolism (31).

Fig. 6 shows how the bioenergetic status and pH of the tumors studied varied during the infusion period. The β-NTP/Pi ratio decreased slightly, but not significantly, at the beginning of the glucose infusion protocol and remained constant thereafter for approximately 1 h (Fig. 6A). No change in the β-NTP/Pi was observed in untreated controls or in tumors whose hosts were infused with saline. At the end of the infusion, pHi was 7.30±0.03 and 7.03±0.19 for animals infused with saline and glucose, respectively. A slight but insignificant, 0.30 pH unit, intracellular acidification occurred during glucose infusion. The pHe was constant during the infusion in both the groups, being 6.76±0.04 and 6.71±0.03 for the glucose and saline infused groups, respectively (Fig. 6B).

Discussion

The modification of tissue oxygenation for purposes of improving radiation response has been studied extensively in pre-clinical tumor models. The three parameters that can be manipulated to this end are tumor blood flow, red blood cell oxygen carrying capacity and tissue oxygen consumption. In elegant modeling studies based on data obtained from a rodent window chamber model, Secomb et al (32) have shown that the most efficient method of changing tumor oxygenation is by modifying cellular respiration. The possibility of inducing the Crabtree effect has encouraged the investigation of hyperglycemia as a means of decreasing or eliminating the number of hypoxic, and, therefore, radiation resistant cells in tumors (33). Glucose infusion has been studied as a clinically relevant and safe method of modifying tumor microenvironment. It has been hypothesized that hyperglycemia could be exploited as a means of positively influencing treatment response, in particular in association with radiation (34) and/or hyperthermia (9).

Since the biological effects of glucose are varied, the impact of hyperglycemia on radiation response should depend on the overall effect of glucose on tumor oxygenation. This, in turn, is a function of the balance of often competing physiologic changes. In addition to the possibility of inducing a Crabtree effect, glucose administration can affect tumor blood flow and the concentrations of H$^+$ and lactate. The direction and degree of tumor blood flow is dependent on a number of factors. Intraperitoneal administration of hypertonic glucose may induce osmotic water shifts from the vascular compartment to the intraperitoneal cavity, leading to a hypovolemic hemococoncentration, a decrease in the cardiac output and a decrease in the tumor blood flow (TBF) (35-39). The effects of a decrease in TBF may be sustained by an increase in the blood viscosity resulting from an increase in red blood cell rigidity. The latter also modifies oxygen carrying capacity and may decrease tumor oxygenation.
Glucose induced changes in lactate and H+ concentration may also modify radiation response. Changes in pH have been shown to affect potentially lethal damage repair in vitro (40), and prolonged exposure of CHO cells to lactate in vitro has been reported to cause radiation sensitization (41). Because of the many possible effects of hyperglycemia, it is not surprising that there is considerable controversy about the efficacy of this maneuver for radiation sensitization. Very few studies have directly evaluated the effects of hyperglycemia on radiation response in vivo. Chaplin and Horsman (8) studied the effect of radiation following glucose administration in mice bearing Lewis lung or EMT6 mammary tumors. Neither acute (single IP injection of 8 mg/kg) nor chronic (multiple IP injections of 6 mg/g glucose plus glucose introduced into the drinking water) administrations of glucose increased the radiation response of either tumor type. In another study, the effect of hyperglycemia on the radiation response of mice bearing a F35A-II tumor on their foot was studied (6). Pre-administration of glucose increased the hypoxic and chronically hypoxic cell fractions without altering the slope of the dose-response curve. On the other hand, glucose administered intra-peritoneally to mice bearing intracranially implanted anaplastic astrocytomas increased mean survival time (42). In mice with transplantable sarcoma 45 and sarcoma M1, glucose injections of 6-17.5 g/kg were shown to enhance radiation response, especially in small tumors (43). However, at higher or lower glucose doses, this effect was not observed.

The current study is unique in that we have used a clinically and experimentally relevant infusion protocol to attain a steady state blood glucose level. Since modification of tumor oxygenation was the major goal of the study, this endpoint was measured using two independent techniques, EF5 binding and Eppendorf needle electrode. These studies demonstrated that the tumors were hypoxic preceding treatment, a necessary requirement in order to observe the Crabtree effect, if present. Detailed studies using multiple techniques documented that hyperglycemia did not cause tumor oxygenation in this system and, therefore, radiation response (using the paired survival and regrowth assays) was not improved, consistent with the oxygenation measurements. It should be noted that the earliest reports on the RIF-1 tumor indicated that this tumor had a low hypoxic fraction (13-17), while more recent studies are consistent with our findings (18).

The effect of glucose infusion on the energetic status of RIF-1 tumors was followed non-invasively using 31P NMR spectroscopy as phosphorus NMR spectroscopy has been shown to monitor phospholipid and energy metabolism as well as pH and pH, of tumors (27). Previous studies have evaluated the ability of 31P MRS to detect differences in the hypoxic fraction or radiosensitivity of tumor cells. Although the data by Rofstad et al (44) indicated a relationship between hypoxic fraction and tumor bioenergetic status as well as tumor HbO2 saturation status (15) within a specific tumor line during tumor growth, there was no correlation between hypoxic fraction and tumor bioenergetic status or tumor HbO2 saturation status across the tumor lines studied (RIF-1 and 3 ovarian tumors). Studies by Koutcher et al (45), demonstrated an approximately linear relationship between the decrease in the values for radiation dose and the increase in PCr/Pi. In a review of this literature, Steen concluded that 31P MR spectroscopy is directly sensitive to metabolic hypoxia in tumors, but it is only indirectly sensitive to radiobiologic hypoxia in tumors (46). Our studies demonstrated minimal changes in tumor energetic or pH status, despite measurable hyperglycemia. The decrease in the pH after glucose loading (0.3 pH unit) and the slight drop in the β-NTP/Pi ratio after the beginning of the glucose infusion may have been either due to a shift from oxidative metabolism to glycolysis, a decrease of the hematocrit, a change in red blood cell rigidity or a decrease in TBF (47). The β-NTP/Pi and PCr/Pi ratios were not modified during saline infusion. Further studies to resolve these issues could include measuring absolute concentrations of metabolites (48).

In conclusion, employing independent endpoints, we have shown that subcutaneous RIF-1 tumors in mice, under the anesthetic conditions described, are hypoxic and radiation resistant, and hyperglycemia induced by intravenous glucose infusion does not elicit a Crabtree effect in modulating radiation response, energy status or tumor pH.

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