Intracellular Acidification of Human Melanoma Xenografts by the Respiratory Inhibitor m-Iodobenzylguanidine Plus Hyperglycemia: A 31P Magnetic Resonance Spectroscopy Study

Rong Zhou, Navin Bansal, Dennis B. Leeper, and Jerry D. Glickson

Department of Radiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104 [R. Z., N. B., J. D. G.], and Department of Radiation Oncology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 [D. B. L.]

ABSTRACT

In vivo 31P magnetic resonance spectroscopy demonstrates that human melanoma xenografts can be significantly acidified by induction of hyperglycemia combined with administration of m-iodobenzylguanidine (MIBG), an inhibitor of mitochondrial respiration. In melanoma xenografts (≤8 mm diameter), intracellular pH (pHi, measured by the chemical shift of the P, resonance) and extracellular pH (pHe, measured with 3-aminopropylphosphonate) was reduced by less than 0.2 unit during i.v. infusion of glucose for 40 min. Administration of MIBG (30 mg/kg) under hyperglycemic conditions (26 mm) reduced tumor pHi and pHe by −0.4 (P < 0.001) and −0.6 (P < 0.001) unit, respectively; coincidentally, the nucleoside triphosphates:Pi ratio decreased −60% (P < 0.0004) relative to the baseline level. Minimal changes in pHi and pHe and a small decrease in nucleoside triphosphates:Pi ratio (26%, P = 0.2) were observed in liver in response to MIBG plus hyperglycemia. These results suggest that under normoglycemic and hyperglycemic conditions, small human melanoma xenografts (≤8 mm) may exhibit a relatively high level of oxidative phosphorylation that may be blocked by MIBG. The acidification may result from increased lactate production as a direct effect of MIBG inhibition of respiration in mitochondria of tumor cells, or through indirect systemic effects, which remain to be identified. The synergetic effects of MIBG and hyperglycemia result in significant acidification of the tumor and a decrease in tumor bioenergetic status, and the effects are largely selective for tumors in comparison with normal tissues.

INTRODUCTION

Melanoma is the most rapidly increasing form of cancer in the United States, exhibiting a 4% increase per year since 1973 (1). Hyperthermia is an attractive therapy for treating superficial depositions of this skin cancer (2). Recent clinical trials demonstrated a substantial benefit of hyperthermia as adjuvant to radiotherapy for treatment of recurrent and metastatic melanoma (3). Extracellular acidification of tumors has been demonstrated to enhance the effect of hyperthermia experimentally and clinically (4–6) and to inhibit development of thermotolerance in cultured tumor cells (7). Furthermore, intracellular and/or extracellular acidification enhances the cytotoxicity of certain chemotherapeutic agents to tumor cells in vitro and in vivo (8–11).

In contrast to normal cells, malignant cells have a higher tendency to use glycolysis as their key pathway of energy production, converting glucose to lactate even under aerobic conditions (12, 13). Administration of glucose reduces tumor pH to various degrees in many rodent and human tumors (14–16). The variability in tumor acidification may be attributed to a dose-dependent response to glucose, to reduction in tumor blood flow, and/or to different levels of oxidative metabolism in various tumors. Although the presence of excessive glucose is necessary for increasing pyruvate production, significant tumor acidification would not be achieved if the tumor exhibited a high level of oxidative metabolism, which competes with lactate production for the available pyruvate. Therefore, inhibition of oxidative phosphorylation at site-1 by administration of MIBG has been proposed as a method of increasing lactate production by tumor cells, hence enhancing tumor acidification in vitro and in vivo (17–20).

Radiolabeled MIBG, an analogue of the neurotransmitter norepinephrine, is concentrated in neuroectodermal tumors derived from adrenergic tissues and has been used in clinical diagnosis and therapy of these tumors (21). By inhibiting oxidative phosphorylation, MIBG would be expected to divert glycolytic flux from the TCA cycle into lactate production, resulting in increased uptake of glucose and enhanced glycolytic flux (17). Kuin et al. (19) showed that the extracellular pH (pHe) of radiation-induced fibrosarcoma-1 (RIF-1) tumors was reduced by 0.24 pH unit during hyperglycemia alone (14 mm blood glucose), whereas a 0.55 pH unit reduction was observed when 100 mg/kg MIBG was combined with hyperglycemia. Whereas MIBG treatment induced a 2- to 3-fold stimulation of 18F-deoxyglucose uptake in tumors, MIBG also reduced up to 5-fold the amount of glucose required to maintain blood glucose levels at 14 mm (i.e., it acted as an analogue of norepinephrine, releasing glucose from glycogen stores in the liver). Therefore, a dual mechanism of MIBG has been proposed in that it blocks respiration via its biochemical mechanism at the cellular level and promotes glucose availability to the tumor via its systemic effect in a stress-related, sympathomimetic response (19).

For hyperthermia sensitization, reduction of pHi is more critical than reduction of pHe (22–25). Acute reduction of pHe sensitizes tumor cells to heat by virtue of an associated reduction in pHi. By using P, (26, 27) and 3-APP (28) resonances as intra- and extracellular pH markers, respectively, 31P MR spectroscopy allows noninvasive and simultaneous monitoring of pHi and pHe as well as the bioenergetic status in live animals. In most normal tissues, the P, resonance is generally thought to be a marker of pHi. For tumors, if the extracellular volume does not exceed 55%, the P, peak primarily reflects pH, However, in large tumors that exhibit extensive necrosis, the extracellular P, may become abnormally high and the P, resonance would reflect pH, (29). Therefore, we used relatively small xenografts, in which the absence of extensive necrosis was confirmed by histological analysis. Hence for these tumors, P, was a reliable indicator of pH, Smaller tumors also tend to have a higher perfusion rate and greater metabolic activity than larger tumors (30). The longer doubling time (~5 days) and lower tumor-body burden of these human melanoma xenografts make this tumor model more pertinent to the clinical situation than many murine tumor models.

The present study was undertaken to evaluate the feasibility of selective acidification of an early passage human melanoma xenograft (DB-1) by administration of MIBG under hyperglycemic conditions.
Using $^{31}$P MR spectroscopy, we have measured pH$_i$ and pH$_e$ profiles, as well as changes in the bioenergetic status of melanoma xenografts in response to hyperglycemia and MIBG. Comparative data on normal liver, skeletal muscle, and brain have been obtained to determine whether MIBG has a selective effects on tumors and possible mechanisms underlying this selectivity have also been considered.

**MATERIALS AND METHODS**

**Materials.** MIBG and 3-APP were purchased from Sigma. Male ICR-SCID mice were obtained from Taconic Farms, Germantown, NY, at 5–8 weeks of age. They were housed in microisolator cages and had access to water and autoclaved mouse chow ad libitum. MIBG was dissolved in PBS (4 mg/ml) and was administered i.p. at a dose of 30 mg/kg. Forty-five minutes before MR data acquisition, 0.1 ml of 145 mg/ml solution of 3-APP (dissolved in saline and pH adjusted to 7) was administered i.p. (580 mg/kg).

**Human Melanoma Xenografts in SCID Mice.** Human melanoma (DB-1) cells were obtained from a patient of Dr. David Berd at Thomas Jefferson University Hospital. The metastatic melanoma was excised before administration of any treatment. Cells were prepared from the tumor and cryopreserved after the 16th passage. For tumor inoculation, $2.0 \times 10^6$ melanoma cells in 0.10 ml of PBS were injected s.c. into the right thigh of each animal (34 ± 2 g). Melanoma xenografts were allowed to grow until they reached 7–8 mm in diameter along the long axis of the tumor. All animal protocols were approved by the Institutional Animal Care and Use Committees at the respective institutions.

**Animal Preparation.** Tumor-bearing mice were anesthetized with ketamine/acepromazine at 660.4 mg/kg, a mixture that minimally perturbs blood glucose level or tumor blood flow (31). Animals were maintained under sedation by administration of one-half of the initial dose of the anesthetics at 30–55 min intervals. A tail vein catheter was placed for i.v. infusion of glucose to maintain the blood glucose level between 25 and 27 mm. Intraperitoneal catheters were placed for delivery of additional anesthetics and MIBG during the MR experiment without removing the animal from the magnet.

Changes in pH in response to hyperglycemia and MIBG were examined in normal brain, skeletal muscle, and liver of SCID mice. An MR surface coil (described below) was placed over the shaved skull for data acquisition from muscle and brain, respectively. For MR experiments on liver, a 1-cm incision was made into the abdomen of anesthetized animals to expose the liver, over which the surface coil was placed. A plastic spacer was inserted between the coil and the overlying skin flap to isolate MR signals from the liver from those of skin.

**Glucose Infusion.** A stock solution of d-glucose (600 mm) was delivered through a tail vein catheter with a syringe pump (Harvard Apparatus, Holliston, MA). An infusion protocol that yielded a glucose concentration of 26 ± 3 mm (mean ± SD) was developed in separate bench top experiments using weight- and age-matched tumor-bearing cohorts (which were not used in the MR studies). Blood samples were obtained from the orbital sinus of the mice, and the blood glucose level was determined from Chemstrips mechanically read in a blood glucose analyzer (Accu-Chek, Boehringer Mannheim Corp., Indianapolis, IN). This infusion protocol was used in subsequent MR experiments.

**MR Experiment and pH Estimation.** The mouse was placed on a water-circulating blanket (42°C) to maintain core temperature at 37°C (measured with a thermocouple outside of the magnet). The MR studies were performed on a GE 9.4T/8.9-cm vertical bore Omega system. *In vivo* $^{31}$P spectra were acquired with a homemade single turn solenoidal surface coil (10 mm in diameter) placed over the tumor with the following parameters: 128 scans with an interpulse delay of 2 s; an rf pulse width of 15–20 μs, corresponding approximately to a 90-degree flip angle; 15 kHz sweep width; and 2048 data points. Data were processed on a SUN computer using 25-Hz line broadening to increase the signal/noise ratio and a convolution difference routine to minimize the broad phospholipid peak underneath the spectrum. A Lorentzian line-fitting routine (Spectrum Analysis Tool) provided by the manufacturer was used to resolve peaks and measure their areas. No corrections for partial saturation of resonances were applied.

Intracellular and extracellular pH were determined from the Henderson-Hasselbalch equation using the chemical shifts of P$_i$ and 3-APP, respectively, referenced to α-NTP resonances. For pH$_i$, the pK$_a$ of 6.91, limiting acid chemical shift of 34.30 ppm, and base chemical shift of 31.11 ppm are used, and for pH$_e$, these parameters are 6.65, 13.25, and 10.85 ppm, respectively (26, 32, 33). The ratio of the peak areas of the β-NTP and P$_i$ resonances (NTP:P$_i$) served as an index of tumor bioenergetic status (26). The change of NTP:P$_i$ ratio relative to its baseline value was determined during hyperglycemia and MIBG intervention for each animal.

**Statistics.** Friedman repeated measures ANOVA (SigmaStat) was used for statistical analysis. Raw data of pH values or NTP:P$_i$ ratios during baseline, hyperglycemia alone, and MIBG administration under hyperglycemic conditions were compared. $P < 0.05$ was considered statistically significant.

**RESULTS**

A representative $^{31}$P MR spectrum from a small xenograft (≤8 mm) is shown in Fig. 1 (*upper spectrum*). Histological analysis of the tumors excised at the end of the MR experiments indicated an absence of necrosis in these xenografts. A $^{31}$P spectrum from a larger xenograft (≥13 mm) is also shown in Fig. 1 (*lower spectrum*). The larger tumors have higher P$_i$ and lower NTP levels, and they usually exhibit extensive necrosis. The absence of a phosphocreatine peak in spectra of some of the tumors suggests that the phosphocreatine signal probably originated either from muscle adjacent to the tumor or from overlying skin (34).

The pH profiles of the xenografts during hyperglycemia and after MIBG administration are plotted in Fig. 2 and summarized in Table 1. The blood glucose concentration estimated from age- and weight-matched cohorts is also presented (no MIBG was administered in this bench top experiment). The blood glucose level was elevated above 300 mg/dl within 10 min after initiation of infusion and was maintained at 473 ± 17 mg/dl (26 ± 1 mm) for >2 h during continuous i.v. infusion. The amount of glucose administered to animals during 100 min of infusion was ~3.8 g/kg in ~1.2 ml. Separate bench top experiments were conducted on four mice in which MIBG (30 mg/kg) was injected during glucose infusion. No significant change of blood glucose concentration was observed in response to MIBG under hyperglycemic conditions (data not shown). It is possible that the preexisting hyperglycemia induced by glucose infusion masked a small elevation of blood glucose level by MIBG.

MR experiments showed that pH$_i$ and pH$_e$ of the tumors were altered by <0.2 unit during 40 min of glucose infusion (Fig. 2). In response...
A slight decrease in NTP:P_i was recorded during hyperglycemia alone, and an accumulated decrease of 26 ± 8% (P = 0.2) was noted in response to MIBG combined with hyperglycemia.

**DISCUSSION**

MIBG combined with hyperglycemia significantly acidified human melanoma xenografts by reducing pH_i and pH_e by ~0.4 and ~0.6 unit, respectively. Here we report for the first time that MIBG decreases pH_i in vivo, a more critical parameter for thermosensitization than pH_e. Intracellular acidification is also responsible for the potentiation of cytotoxicity of certain chemotherapeutic agents, such as platinum-containing drugs and alkylating agents (8–11). Response to hyperthermia is closely related to the extent of tumor pH reduction (35). For example, Hiraoka and Hahn (36) showed that a reduction of tumor pH_i by 0.15 unit had little effect on hyperthermia response, a 0.25-unit reduction increased response by a factor of 1.5, and a 0.35-unit reduction increased response by a factor of 4. Therefore, with a ~0.6 unit decrease in pH_i and a ~0.4 decrease in pH_e in DB-1 melanoma xenografts, significant thermosensitization could be expected. Furthermore, the significant reduction in tumor bioenergetic status would also sensitize tumor cells to hyperthermia.

Small human melanoma xenografts may be uniformly well perfused and have a relatively high level of oxidative phosphorylation, which can be blocked by MIBG. This is consistent with the observation that pH_e was reduced by MIBG in the peripheral regions of RIF-1 tumors, where the density of perfused vessels is 42% higher than in central regions of the tumor (19). The marked decrease of NTP:P_i, pH_i, and pH_e of melanoma xenografts following MIBG + hyperglycemia suggests an increase of glycolytic flux, which results in an increase of lactate production, as well as a decrease in NTP production by the tumor. Because only the ratio of NTP to P_i was measured, it is conceivable that P_i increased whereas NTP remained constant. This is highly unlikely because P_i is usually generated by NTP hydrolysis; however, this remote possibility can be excluded by absolute quantitation of NTP concentrations, as has been done by our laboratory in the past (37). Such experiments are planned in the future. A decrease in steady state levels of NTP could result from inhibition of respiration by MIBG; this would also lead to accumulation of P_i. Both lactate production and NTP hydrolysis generate protons, leading to tumor acidification. The decrease in tumor bioenergetic status can be attributed to the lower efficiency of glycolysis as a pathway for NTP synthesis compared to the TCA cycle (2 versus 36 NTPs per glucose molecule, respectively).

There is, however, an alternate mechanism that could contribute to the observed data. Tissues with higher levels of oxidative metabolism usually exhibit higher NTP:P_i values; in normal tissues, these ratios precipitously decrease during ischemia or hypoxia. The pattern of decreases in pH_i and pH_e in conjunction with a decrease in NTP:P_i is also reminiscent of ischemia. Preliminary pharmacokinetic measurements with 131I-MIBG suggest that little radiolabeled MIBG is reaching the tumor; most of it goes to the liver and kidneys with significant 131I accumulation in the thyroid gland (suggesting metabolism of MIBG)4. Preliminary results from their study also suggest that administration of MIBG, at least under some conditions, appears to induce a decrease in blood pressure, presumably because MIBG could act as an antagonist of norepinephrine in the vascular smooth muscle.4 A decrease in blood pressure could produce a steal effect, leading to decreased tumor perfusion. This could explain the decrease in bioenergetic status and pH_i and pH_e of tumors in this study. Similar effects have been observed with hydralazine (38). This systemic mechanism could explain the apparent specificity of acidification and bioenergetic decline to the tumor, because most normal tissues (with the possible

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ACIDIFICATION OF HUMAN MELANOMA XENOGRAFTS

Table 1 Summary of pH in tumors and normal tissues measured by MR in response to hyperglycemia and MIBG

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Melanoma xenografts (N = 7)</th>
<th>Normal tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH&lt;sub&gt;i&lt;/sub&gt;</td>
<td>pH&lt;sub&gt;e&lt;/sub&gt;</td>
</tr>
<tr>
<td>None</td>
<td>7.03 ± 0.03</td>
<td>6.95 ± 0.07</td>
</tr>
<tr>
<td>Hyperglycemia alone&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.98 ± 0.06</td>
<td>6.88 ± 0.02</td>
</tr>
<tr>
<td>Hyperglycemia + MIBG&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.67 ± 0.08&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.36 ± 0.13&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> The pH values were determined by <sup>31</sup>P MR spectroscopy as described in "Materials and Methods."
<sup>b</sup> Data are presented as mean ± SEM.
<sup>c</sup> pH measured at 40 min after initiation of glucose infusion.
<sup>d</sup> pH measured at 50 min after MIBG injection.
<sup>e</sup> pH<sub>i</sub> is not available due to inaccessibility of 3-APP to brain.
<sup>f</sup> P < 0.001 compared to nontreatment level.

Table 2 Tumor pH<sub>e</sub> measured by needle electrode during glucose infusion<sup>a</sup>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline</th>
<th>1 h</th>
<th>2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>166 ± 22</td>
<td>477 ± 58</td>
<td>489 ± 65</td>
</tr>
<tr>
<td>pH&lt;sub&gt;e&lt;/sub&gt;</td>
<td>6.60 ± 0.06</td>
<td>6.49 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.42 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> The needle pH electrode (20 gauge, manufactured by Dr. S. Agulian, Hamden, CT) was inserted ~4 mm into the tumor (≤8 mm), whereas hyperglycemia was induced by i.v. infusion of glucose (600 mM stock solution). Blood glucose concentration was determined from blood samples drawn from the orbital sinus. Data are presented as mean ± SE (n = 4).
<sup>b</sup> P > 0.05 compared with baseline level.

No acute or cumulative toxicity was observed in liver, kidney, and other organs in response to oral administration of MIBG to mice at 40 mg/kg for 5 consecutive days (42). The maximal effect of MIBG under hyperglycemic condition occurred ~50 min after MIBG administration (39), and no pH change was observed 3 h after MIBG injection (19), suggesting that intracellular acidification of tumors induced by MIBG is most likely transient, probably because tumor cells are able to maintain pH homeostasis by H<sup>+</sup> extrusion mechanisms. We are now examining the possibility that tumor acidification induced by MIBG plus hyperglycemia can be enhanced and maintained longer by inhibition of membrane Na<sup>+</sup>/H<sup>+</sup> and/or HCO<sub>3</sub>⁻/Cl<sup>⁻</sup> exchange mechanisms (43).

MIBG at normoglycemia has no effect on extracellular pH of DB-1 xenografts (39). These observations are more consistent with the first mechanism (inhibition of respiration). In addition, the 26% decline in NTP:Pi of the liver may be substantial, although the dose of MIBG used was well tolerated (see below). Whereas a ~12% fluctuation in basal NTP:Pi level measured by MR spectroscopy could be expected (40), a 26% decrease in response to MIBG is well above this level. The bioenergetic effect of MIBG on the liver is consistent with the preliminary observation that MIBG accumulates in this organ. The liver is, however, a very large organ; hence, being distributed over a large mass may ameliorate the toxic effect of MIBG on the liver.

Stimulation of lactate production in tumors by MIBG is also being examined by <sup>1</sup>H MR spectroscopy using lipid and water suppression MR pulse sequence developed in our laboratory (41). Preliminary results from a human melanoma xenograft in SCID mice showed that during normoglycemia or hyperglycemia tumor lactate was not detected. This suggests a high level of oxidative phosphorylation. Administration of MIBG under hyperglycemic conditions induced an increase in tumor lactate detectable by <sup>1</sup>H MR spectroscopy. This observation would be consistent with either inhibition of respiration or a steal effect.

The dose of MIBG used in this study (30 mg/kg) was tolerated well in SCID mice. The animals were not lethargic, and no weight loss was observed for 1 week after administration, suggesting minimal toxicity.

<sup>5</sup> J. D. Glickson, Q. He, and D. B. Leeper, unpublished results.

Fig. 3. The pH<sub>e</sub> profile of normal tissues (liver, muscle, and brain) in response to hyperglycemia (473 mg/dl) and MIBG (30 mg/kg). ●, liver (n = 3); □, muscle (n = 1); ▲, brain (n = 1). Data are presented as mean ± SEM. For clarity, pH<sub>e</sub> values were not plotted here but are summarized in Table 1.

Fig. 4. Changes of NTP:Pi (ratio of peak area) relative to baseline level in melanoma xenografts (●, n = 6) and in livers (▲, n = 3) in response to hyperglycemia (473 ± 17 mg/dl) and i.p. injection of MIBG (30 mg/kg). Data are presented as mean ± SEM.
malignancy xenografts measured by pH electrode. No response or a much smaller decrease of tumor pH was also observed in response to MIBG alone compared with MIBG + hyperglycemia in other human and rodent tumors (18). Hyperglycemia (473 mg/dl) alone had limited acidifying effect on DB-1 human melanoma xenografts (Table 2). Therefore, our results suggest the synergistic effect of MIBG and hyperglycemia and are consistent with related studies (19). Coadministration of MIBG appeared to achieve tumor acidification at moderate hyperglycemia, avoiding the adverse effect of severe hyperglycemia.

In conclusion, 31P MRS allows noninvasive and simultaneous monitoring of pH, pO2, and cellular energy status. MIBG plus hyperglycemia can significantly acidify human melanoma xenografts in which necrosis is absent, whereas it has only a minimal effect on pH in livers, skeletal muscle and brain. The data suggest that MIBG combined with hyperglycemia could be a nontoxic and effective mechanism for intracellular acidification of tumors, and, hence, for enhancing the effect of hyperthermia. The mechanism underlying tumor acidification by MIBG under hyperglycemic conditions remains to be elucidated. At least two possible mechanisms have been presented, one involving a direct cellular effect on tumor energy metabolism by inhibiting respiration and the other involving indirect induction of ischemia in the tumor.

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