Acid Production in Glycolysis-impaired Tumors Provides New Insights into Tumor Metabolism

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ABSTRACT

Purpose: Low extracellular pH is a hallmark of solid tumors. It has long been thought that this acidity is mainly attributable to the production of lactic acid. In this study, we tested the hypothesis that lactate is not the only source of acidification in solid tumors and explored the potential mechanisms underlying these often-observed high rates of acid production.

Experimental Design: We compared the metabolic profiles of glycolysis-impaired (phosphoglucose isomerase-deficient) and parental cells in both in vitro and two in vivo models (dorsal skinfold chamber and Gullino chamber).

Results: We demonstrated that CO₂, in addition to lactic acid, was a significant source of acidity in tumors. We also found evidence supporting the hypothesis that tumor cells rely on glutaminolysis for energy production and that the pentose phosphate pathway is highly active within tumor cells. Our results also suggest that the tricarboxylic acid cycle is saturable and that different metabolic pathways are activated to provide for energy production and biosynthesis.

Conclusions: These results are consistent with the paradigm that tumor metabolism is determined mainly by substrate availability and not by the metabolic demand of tumor cells per se. In particular, it appears that the local glucose and oxygen availabilities each independently affect tumor acidity. These findings have significant implications for cancer treatment.

INTRODUCTION

Low interstitial pH is a well-established pathophysiological characteristic of solid tumors (1–9). This intratumoral acidity is therapeutically important because it interacts with various therapies, including chemotherapy, hyperthermia, and photodynamic therapy (2, 4, 10–18). Recent interest in pH stems from the ability of low pH to up-regulate various angiogenic molecules, e.g., vascular endothelial growth factor and interleukin-8 (19, 20). It has long been thought that lactic acid is the main source of tumor acidity. This conjecture is supported by numerous studies showing that experimentally induced hyperglycemia acidifies the tumor microenvironment (21–23) after an increase in the glycolytic rate (24). The diagnostic importance of lactate has been emphasized in recent studies showing a correlation between high lactate concentration and metastatic incidence (25, 26). However, lactic acid is probably not the only source of acid in tumors. Studies by Gullino et al. (27) have shown that the TIF of numerous tumor types contains high levels of CO₂, which is another potential source of acidity. Additionally, studies by Newell et al. (28) and Yamagata et al. (29) demonstrated that solid tumors derived from glycolysis-impaired cells with a defect in either PGI or lactate dehydrogenase, respectively, were as acidic as their parental counterparts, although they produced negligible amounts of lactate.

In this report, we test the hypothesis that lactate is not the only source of acidification in solid tumors and describe a potential mechanism to explain the observed high rates of acid production. Using glycolysis-impaired tumor cells and their parental counterpart (28), we analyzed the metabolic microenvironment with a combined in vitro and in vivo approach. We used two in vivo models: (a) the dorsal skinfold chamber preparation (30), which provides a transparent window for noninvasive spatial and temporal measurements of metabolic parameters (pH and pO₂), and (b) the Gullino chamber (27, 31), which allows sampling of TIF secreted by solid tumors and an analysis of its metabolic characteristics (pH, CO₂, glucose, lactate, and HCO₃⁻ content). Our results indicate that lactic acid is not the only source of acid in tumors and that CO₂ may be a significant source of acid production. Our results further suggest that tumors partially rely on glutaminolysis and that the PPP is highly active. Our observations also imply that the TCA cycle is

The abbreviations used are: TIF, tumor interstitial fluid; pO₂, partial pressure of oxygen; PGI, phosphoglucose isomerase; PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle; SCID, severe combined immunodeficient; FRIM, fluorescence ratio imaging; pCO₂, partial pressure of carbon dioxide.
saturable, potentially explaining this observed decoupling of acid and lactate production.

MATERIALS AND METHODS

Cell Lines. Both parental (ras94\textsuperscript{+}) and variant (ras94\textsuperscript{−}) lines, ras-transfected Chinese hamster lung fibroblasts, were a generous gift of Dr. I. Tannock (Ontario Cancer Institute, Toronto, Canada). The variant cell line was selected as described previously (32). Variant cells have a defect in the glycolytic enzyme PGI, with ≤1% activity compared with parental cells (28, 32). These original lines were passaged seven times, producing the glycolysis-competent ras5B\textsuperscript{+} and glycolysis-deficient 7ras3\textsuperscript{−} cell lines.

Glucose Consumption, Lactate and H\textsuperscript{+} Production, and Cell Growth in vitro. Parental and variant cell lines were grown to near confluence in α-MEM culture medium buffered with 25 mM NaHCO\textsubscript{3} and supplemented with 10% fetal bovine serum. Cultures were then rinsed with PBS and provided with fresh culture medium (t = 0). Glucose and lactate were subsequently assayed in conditioned medium, which was sampled (80 μl) every 2 h (from t = 0 to t = 6 h); standard assay kits (Sigma Chemical Co.) and a spectrophotometer (Model Lambda 3; Perkin-Elmer, Oak Brook, IL) were used. H\textsuperscript{+} production was determined in near-confluent cultures that were placed in bicarbonate-free α-MEM lightly buffered with 3 mM HEPES (initial pH 7.3) and gassed with humidified 100% air. Conditioned medium was sampled (80 μl) every 2 h and transferred to a blood gas analyzer (Model ABL 300; Radiometer, Copenhagen, Denmark) to yield pH values. Cell growth was determined by monitoring the number of cells per unit surface area. Cells were seeded at a low density (100 cells/mm\textsuperscript{2}) and counted on a daily basis, from day 0 to day 3, in five different fields/well. Glucose, lactate, pH, and cell growth were all determined in the absence or presence (2, 20, and 100 mM) of mannoheptulose (Fluka Chemika), a specific inhibitor of glucokinase activity (33).

Measurement of pH and glucose-induced pH Changes in vivo: The Dorsal Skinfold Chamber Model. Experiments were performed in SCID mice (6–8 weeks of age; 25–30 g) bred and housed in a defined flora animal colony. The dorsal skinfold chamber was surgically implanted under anesthesia (75 mg of ketamine and 25 mg of xylazine per kg s.c.), as described previously (30). After a 2-day recovery period, ras-transfected tumors (both parental and variant) were grown by implanting tumor chunks (≈1 mm\textsuperscript{3}) in the chamber. Chunks were derived from primary tumors grown for 2–3 weeks in the hind flanks of SCID mice. Tumor growth (en face diameter) and microcirculation in the dorsal skinfold chamber were monitored through the glass coverslip, using an intravital microscope station (9). pH experiments were performed 20–30 days after seeding of the chamber, when tumors had reached an en face diameter of 4.0 ± 0.7 mm. Intersitial pH was measured before and after hyperglycemia (0.45 ml; 6 g/kg i.v.) in several tumor locations, using FRIM with the pH-sensitive probe 2’7’-bis-(2-carboxyethyl)-5,6-carboxyfluorescein in vivo. FRIM is a noninvasive, intravital microscopic technique with high spatial and temporal resolution that we developed in our laboratory (7–9, 21). Partial confocal effects were created on the microscope to obtain a lateral spatial resolution of 5 × 5 μm\textsuperscript{2} and a sampling depth of ≤25 μm (9).

Measurement of Blood Flow and Local pO\textsubscript{2}. Blood flow in selected vessels was measured via transillumination and off-line analysis, as described previously (21, 30). The pO\textsubscript{2} was measured noninvasively by phosphorescence quenching microscopy (9). Partial confocal effects on the microscope yielded a lateral spatial resolution of 10 × 10 μm\textsuperscript{2} and a sampling depth of ≤25 μm (9).

Measurement of Lactate, pCO\textsubscript{2}, HCO\textsubscript{3}\textsuperscript{−}, pH, and Glucose in TIF in vitro: The Gullino Chamber Technique (27, 31). A wafer-like chamber with semipermeable walls (0.45 mm cutoff size; Millipore, Bedford, MA) was implanted s.c. in SCID mice. One hundred μl of tumor slurry were seeded on each side of the chamber. As the tumor grew, TIF accumulated within the internal cavity of the chamber. After 7–10 days of growth, the animal was anesthetized, and the TIF (≈400 μl) was immediately sampled by accessing the isolated cavity with a syringe. Eighty-five μl of the extracted TIF were then injected directly into a blood gas analyzer (ABL 300; Radiometer) without exchange with the ambient air. The analyzer simultaneously yielded pCO\textsubscript{2}, HCO\textsubscript{3}\textsuperscript{−} concentration, and pH values for the fluid sample. The remaining TIF was used for subsequent determinations of lactate and glucose by standard assay kits (Sigma) and a spectrophotometer (Lambda 3).

RESULTS

In Vitro Metabolism of Parental and Glycolysis-impaired Cell Lines. Fig. 1 shows the temporal profiles of glucose utilization, lactate production, medium acidification, and cell growth for both the parental ras5B\textsuperscript{+} and the variant,
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In vitro, the lactate production rates of the parental and variant cells were 7.8 ± 1.2 and 0.7 ± 0.2 μmol/h/10^7 cells, respectively (Fig. 1A). The glycolysis-impaired cells consumed a small but measurable amount of glucose compared with the parental line. The consumption rate of the impaired cells was 15% of that of the parental cells: 0.6 ± 0.3 versus 3.9 ± 0.8 μmol/h/10^7 cells, respectively (Fig. 1B). At similar densities, the parental and variant cell lines acidified a lightly buffered medium in 6 h of culture, inducing pH drops of 0.4 and 0.13 pH units, respectively (Fig. 1C). Both cell lines exhibited comparable cellular growth rates (Fig. 1D); however, a considerable time lag was observed with impaired cells before growth became apparent.

The lactate yields from glucose for the original parental and glycolysis-impaired cells (ras94+ and ras94−) and for the lines after seven passages in culture (ras5B+ and 7ras3−) are shown in Fig. 2A. Lactate yields were calculated by dividing the lactate production by the glucose consumption rates. For both the original and cultured groups, the lactate yield was significantly (P < 0.05) lower in the glycolysis-impaired than in the parental cells. The parental cells exhibited lactate yields close to the theoretical maximum of two, whereas the impaired cells had much lower yields, indicating that not all of the consumed glucose was converted into lactate.

The ratio of H3O+ to lactate production is shown in Fig. 2B. This ratio was calculated similarly to the lactate yield; the H3O+ production rate (derived from pH measurements) was divided by the lactate production rate. The ratio of H3O+ to lactate was significantly higher (P < 0.05) in the glycolysis-impaired than the parental cells, indicating that acid is derived from a source other than lactate in the impaired cells.

Pouyssegur et al. (32) demonstrated, with similar cells, that starvation would return the glucose transport activity of the impaired cells to that of the parental cells. However, as shown in Fig. 2 passaging the different cell lines in culture did not significantly alter their metabolic phenotype. After seven passages, the parental ras5B+ and variant 7ras3− cell lines exhibited identical rates of glucose consumption, lactate production, medium acidification, and growth compared with the original ras94+ and ras94− cell lines.

Similar experiments were repeated in the presence of 2, 20, or 100 mM mannoheptulose, a specific inhibitor of the enzyme glucokinase, whose increased activity we hypothesized would accumulate glucose-6-phosphate and would subsequently increase the activity of the PPP. However, none of the concentrations tested had a significant effect on the rates of glucose utilization, lactate production, medium acidification, or cell growth in either the parental or impaired cell lines (Fig. 3) compared with regular culture medium (Fig. 1). The virtual overlap of Figs. 1 and 3 suggests a minimal effect of 20 mM mannoheptulose. More specifically, lactate yield was not significantly affected at any mannoheptulose concentration. At the highest concentration (100 mM) of mannoheptulose, lactate production was decreased 25% for the parental cell line and growth rate was inhibited for both lines. However, at this high concentration, the specificity of mannoheptulose to glucokinase is uncertain.

Growth and Physiological Characteristics in Vivo.

The ras5B+ (parental) and 7ras3− (variant, glycolysis-impaired) lines both produced solid tumors when grown either in the dorsal skinfold chamber or in the hind flanks of SCID mice. At either site, parental and variant tumors exhibited similar growth rates, but a significant time lag (5 ± 1 days) was observed in variant tumors compared with parental tumors (not shown). Both tumor types exhibited heterogeneous vasculatures, with both richly and poorly vascularized areas (Fig. 4A). Blood vessels often showed a tortuous and dilated morphology (Fig. 4B), a suboptimal spatial organization, and heterogeneous blood flow typical of tumors. Blood flow in individual vessels ranged from 0.2 × 10−5 to 25 × 10−5 mm/s in both tumor types. Thus, no gross differences in vascular morphology or blood flow were detected between the parental and variant tumors.
flow were observed between ras5B⁺ (parental) and 7ras3⁻ (variant) tumors. The similarities in the geometry and tortuosity of the two tumor types indicate that compositional differences are attributable to metabolic differences of the tumor cells themselves. However, mean interstitial pO₂, as determined from multiple local measurements by phosphorescence quenching microscopy (see “Materials and Methods”) in 21-day-old tumors, was significantly higher (P < 0.01) in variant (mean ± SD, 28.2 ± 6.3 mm Hg; n = 34 locations; n = 4 animals) versus parental tumors (10.7 ± 6.0 mm Hg; n = 30; n = 4).

Solid Tumors Derived from Parental and Variant Cells Are Both Acidic and Respond to Hyperglycemia by Lowering pH. Temporal and spatial profiles of interstitial pH were measured in tumors grown in the dorsal skinfold chamber, using FRIM (see “Materials and Methods”). In both parental (not shown) and variant (Fig. 5A) tumors, basal pH was spatially heterogeneous and typically ranged from 6.6 to 7.2. The mean interstitial pH of variant tumors was not statistically different (P < 0.308) from the pH of parental tumors: 6.76 ± 0.12 (mean ± SD; n = 36 locations; n = 6 animals) in parental tumors and 6.79 ± 0.18 (n = 42; n = 6) in variant tumors.

After bolus administration of glucose (0.45 ml; 6 g/kg i.v.), interstitial pH responded heterogeneously in different regions within individual tumors as well as between parental (not shown) and variant (Fig. 5A) tumors. A maximum pH drop of 0.3 pH units was measured in some locations 20 min after glucose injection. In some locations, the interstitial pH initially dropped and then returned to baseline levels within 60 min (Fig. 5A, ▲), in some it remained below baseline after initially dropping (Fig. 5A, ◆), and in others no significant response was measured (Fig. 5A, ■).

The temporal pH profiles after hyperglycemia, as averaged over all interstitial locations, are shown in Fig. 5B. The mean interstitial pHs of parental and variant tumors were not significantly different at 20, 40, and 60 min (Fig. 5B). The instantaneous rate of change in pH (the ratio of pH change to time interval; Fig. 5C) was statistically different (P < 0.001) from zero (flat) only for glycolysis-impaired tumors at the 20-min time point. However, the rate of change in pH for the parental tumors at 20 min had a decreasing trend (P < 0.12). These results imply that both tumor types consume glucose and consequentially produce acid, although the variant tumors have a greater metabolic response to glucose than do the parental tumors.

Metabolic Profile of Parental and Variant TIF. To determine the contribution of different metabolites to tumor acidity in vivo, TIF from parental and variant tumors was collected from Gullino chambers, and the lactate, CO₂, HCO₃⁻, H⁺, and glucose concentrations were subsequently measured (see “Materials and Methods”). The interstitial pH of variant
tumors (Table 1). Lactate concentrations in the sera of animals bearing parental versus variant tumors were similar (Table 1); they were 42–43% lower ($P < 0.0001$) than the mean lactate concentration in parental TIF and 20–21% lower ($P < 0.01$) than the lactate concentration in variant TIF. Importantly, CO$_2$ levels, as measured by the pCO$_2$ and the concentration of HCO$_3^-$ in TIF were similar in both tumor types ($P < 0.131$ and $P < 0.248$, respectively; Table 1). In addition, glucose concentrations in the TIF of parental and variant tumors were similar ($P < 0.274$; Table 1).

**DISCUSSION**

**Lactic Acid Is Not the Only Source of Acid in Tumors.** Using a parental, ras-transfected cell line (ras$5B^+$) and a variant, glycolysis-impaired line (7ras$3^-$), we found, *in vitro*, that impairing glycolysis (specifically, reducing PGI activity) reduced the lactate from glucose yield while increasing the ratio of acid to lactate produced (Fig. 2). This occurred despite the fact that 7ras$3^-$ cells, which have a substantially diminished PGI activity (28, 32) and hence diminished “direct” glycolytic activity, consumed less glucose and produced significantly less lactate and H$^+$ ions per cell (Fig. 1). The significant differences in the lactate yield and the produced acid ratio strongly imply (a) that another source of tumor acidification exists (e.g., HCO$_3^-$) and (b) that lactate contributes less to the acidification of glycolysis-impaired cells than parental cells *in vitro*.

These conclusions were confirmed by our observations of tumors grown *in vivo* from ras$5B^+$ and 7ras$3^-$ cells, which exhibited (a) virtually identical levels of acidic pH, as measured independently in TIF samples of s.c. tumors (Table 1) or by FRIM in dorsal skinfold chamber tumors (Fig. 5B), and (b) identical levels of glucose (as measured in TIF samples; Table 1) and similar dynamics of hyperglycemia-induced pH decrease. Lactate levels were consistently lower in the glycolysis-impaired 7ras$3^-$ line than in the parental ras$5B^+$ line (Table 1). These results confirm earlier findings with the same cell lines by Newell et al. (28) and support the idea that lactate, as produced via glycolysis, is not the sole metabolite responsible for an acidic interstitium in solid tumors (28).

Lactate in variant TIF was significantly higher than serum lactate (Table 1), whereas Newell et al. (28) found no significant differences. This may be attributable to differences in the methods used. Newell et al. (28) applied an enzymatic assay to homogenized tumor tissue, whereas we analyzed the interstitial fluid that accumulated in the Gullino chamber over several days; our values, therefore, represent a “cumulative” lactate value. By deriving the 7ras$3^-$ cell line from the original ras$94^-$ line, we also confirmed that acid production was not attributable to a gradual selection of glycolysis-performing cells and, hence, a reversion of the metabolic phenotype under *in vivo* conditions (28, 32).

**CO$_2$ Is a Significant Source of Acid in Solid Tumors.** We hypothesized that CO$_2$ production *in vivo* may significantly contribute to the acidification of tumor interstitium because of the equilibrium between CO$_2$ released into the extracellular space and carbonic acid. However, CO$_2$ levels in TIF samples of ras$5B^+$ and 7ras$3^-$ tumors were similarly high ($76.9 \pm 7.9$ and

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**Fig. 5** A, temporal dynamics and spatial heterogeneity of pH and hyperglycemia-induced pH changes in a representative 21-day-old variant 7ras$3^-$ tumor (dorsal skinfold chamber). Each trace represents a different interstitial location within the tumor. Arrow indicates time of glucose administration ($t = 0$). The profiles for all 7ras$3^-$ and ras$5B^+$ tumors are qualitatively similar. B, average temporal pH profiles after glucose injection in 21-day-old parental (ras$5B^+$; *hatched columns*) and glycolysis-impaired (7ras$3^-$; *open columns*) solid tumors. The mean ± SE (bars) for each time point is shown ($n = 30$ locations, $n = 5$ for ras$5B^+$; $n = 34$, $n = 6$ for 7ras$3^-$). The pH of the glycolysis-impaired tumors dropped significantly (*, $P < 0.02$) after 20 min. C, instantaneous rate of change in pH after glucose injection, which was calculated as change in pH per unit time. Only the rate of pH decrease in the glycolysis-impaired tumors at 20 min was statistically distinct ($P < 0.001$) from zero. The rate of pH decrease for the parental tumors at 20 min had a decreasing trend ($P < 0.12$).
versus H2O2 secretes a "model") our laboratory also found a strong correlation of their microenvironment. Parental tumors may, nevertheless, produce similar characteristics of their microenvironment. Because the glycolysis-impaired 7ras3- tumors, which have negligible PGI activity (28), were able to consume glucose (Table 1 and Fig. 5, B and C), we hypothesize that the PPI has increased activity in these tumors. A present hypothesis (35–37) for mammalian cell glucose consumption is shown in Fig. 6. The PPI is thought to be the primary source of reducing equivalents (NADPH) necessary for the biosynthetic precursors of nucleotides and lipid, which are both necessary for proliferating tumor cells. Increased PPP activity is also beneficial to proliferating tumor cells because it is the sole source of the ribose required for de novo nucleoside synthesis. In the absence of PGI activity, consumed glucose may be metabolized via the PPP because two of its intermediates (fructose-6-phosphate and glyceraldehyde-3-phosphate) feed back into the glycolytic pathway, as shown in Fig. 6. The PPI produces one molecule of CO2 for every glucose molecule, thus contributing to the acidification of the tumor interstitium. In the hypothesized pathway in Fig. 6, five-sixths of the glucose carbons reenter glycolysis to be consumed for energy.

One mechanism by which tumor cells may increase the activity of the PPP is by increasing the glucose-6-phosphate concentration. Of the two enzymes capable of phosphorylating glucose, glucokinase has a higher Km than does hexokinase and is thus less inhibited by high concentrations of glucose-6-phosphate (33). Our data with ras-transfected fibroblasts show that mannheptulose did not have any effect on glucose consumption or lactate and H+ production by parental ras5B+ cells (Fig. 3, A–C), nor did it affect cell growth rates of parental ras5B+ and variant 7ras3- cells (Fig. 3D), except at very high concentration (>100 mM). Thus, glucokinase activity, which may favor glucose-6-phosphate entry into the pentose cycle, does not play a major role in these ras-transfected fibroblasts and is not necessary to sustain the growth of glycolysis-impaired cells.

Glutaminolysis May Significantly Contribute to Lactate Production in Tumors. Although the lactate levels in glycolysis-impaired 7ras3- tumors were lower than in parental ras5B+ tumors, these levels were both higher than in the serum (Table 1). This strongly implies that both tumor types produce lactate. In addition to glucose, another likely precursor of lactate is glutamine. There is ample evidence that tumor cells have high rates of glutaminolysis, i.e., the consumption and partial oxidation of glutamine by half of the TCA cycle (38–40). Under atmospheric oxygen conditions, multicellular tumor spheroids have been shown to grow in glucose-free medium supplemented with various amino acids, including glutamine (40). Additionally, it has been shown that (a) some glycolysis-competent tumors produce more lactate than is theoretically possible from the glucose consumed (34, 41) and that (b) poor correlations have been observed between the spatial distribution of glucose and lactate (41), thus providing further evidence that glutaminolysis can produce a significant fraction of the total lactate produced.

On the other hand, oxygen is required for the production of energy from glutamine, and solid tumors are known to exhibit low oxygen content and significant hypoxic areas (42). We found that mean P02 values were higher in glycolysis-impaired 7ras3- tumors than in parental ras5B+ tumors (see “Results”). Newell et al. (28) also found a significantly reduced hypoxic fraction in solid tumors derived from the same glycolysis-impaired cells compared with tumors derived from parental cells. Thus, glycolysis-impaired 7ras3- tumors may more extensively use glutaminolysis as a source of energy, which may contribute to their observed accumulation of lactate (Table 1).

Saturation of the TCA Cycle. Pouyssegur et al. (32) showed that similarly glycolysis-impaired cells are dependent on respiration for energy. They found that the addition of oligomycin, an ATPase inhibitor, caused rapid death in glycolysis-impaired cells but did not affect parental cells. Our obser-
demonstrated in vitro that local glucose and oxygen availabilities each independently affect tumor acidity. These findings have significant implications for cancer treatment. Present strategies for treating acidic tumors include ionophores (4) and weakly acidic lipid-soluble drugs (Ref. 11, 45; e.g., chlorambucil) that are either more toxic to, or preferentially accumulate in cells in acidic environments. Inhibition of glutaminolysis (e.g., with antisense mRNA for glutaminase) and the PPP (e.g., with 6-aminonicotinamide) have both been shown to regress tumors in vivo (46–48). The dependence of proliferating tumor cell on the PPP and glutaminolysis indicates their potential as tumor-associated targets.

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