Influence of Hypoxia and an Acidic Environment on the Metabolism and Viability of Cultured Cells: Potential Implications for Cell Death in Tumors

Daniela Rotin, Brian Robinson, and Ian F. Tannock

Abstract

Hypoxia and an acidic environment are known to occur in regions of solid tumors and might be involved in the causation of necrosis. The viability and energy metabolism of cells in tissue culture were therefore investigated under hypoxic and/or acidic conditions. Acute exposure of Chinese hamster ovary (CHO) cells or human bladder cancer MGH-U1 cells to hypoxia plus low pH (6.5 to 6.0) was cytotoxic in a time- and pH-dependent manner; surviving fraction was reduced to $10^{-6}$ following a 6-h exposure to hypoxia at pH 6.0. There was no effect on viability when aerobic CHO cells were exposed for 6 h at pH 6.0, or when either cell line was rendered hypoxic for 6 h at pH 7.0; MGH-U1 cells showed slight sensitivity to acidic pH in air. Decrease in viability of CHO cells incubated under acid conditions was observed over the range of oxygen concentrations from 0.2 to 0.05%, similar to the range which causes change in cellular sensitivity to radiation. Glucose consumption and lactate production by both cell lines were inhibited at low pH under both aerobic and hypoxic conditions. Cellular adenosine triphosphate (ATP) levels and the energy charge ([ATP + ½ adenosine diphosphate]/adenosine monophosphate + adenosine diphosphate + ATP) of CHO cells were reduced by about 85% and 25%, respectively, after a 6-h exposure to hypoxia at pH 6.0 but were not influenced by hypoxia or acid pH alone. Inhibition of glycolysis by incubation of CHO cells under hypoxic conditions in the absence of glucose (at pH 7.0) led to a larger fall in cellular ATP and energy charge, but cell survival fell to only $10^{-6}$ at 6 h. These results demonstrate that hypoxia and an acidic environment interact to cause marked toxicity. A decrease in energy charge of the cells may contribute to loss of viability, but additional mechanisms appear to be involved.

Introduction

Cell death occurs in most solid tumors, but underlying mechanisms remain poorly understood. The edge of a necrotic region in a tumor is sometimes found to be parallel to a neighboring blood vessel (1–3), suggesting that one cause of cell death may be limited diffusion of essential nutrients to the tumor cells or inadequate removal of catabolites by the vascular system. The distance between capillaries and the edge of a necrotic region (typically 100 to 200 μm) (1–3) is consistent with estimates of the diffusion distance for oxygen in tissue (4). Evidence from radiobiological experiments that most solid tumors contain hypoxic cells (4) and the observation that the distance separating necrotic regions from blood vessels was smaller in tumors of mice that were placed in an oxygen-deficient environment than in mice breathing air (5) are consistent with the hypothesis that hypoxia may contribute to cell death.

It is unlikely that hypoxia is the sole cause of cell death in solid tumors. We and others (6) have observed that cultured cells may survive for periods of 24 h or longer when placed in a hypoxic environment, provided that the supply of other nutrients is maintained. Studies of spheroids (i.e., multicellular aggregates of cells which may grow in culture) have demonstrated the formation of a necrotic center in the absence of severe hypoxia (7). The thickness of the viable rim of spheroids appears to depend on limited diffusion of glucose as well as oxygen (8, 9), and other factors probably contribute to cell death.

Hypoxic cells are dependent on anaerobic glycolysis to supply their energy requirements; indeed, hypoxia is known to increase the activity of all 11 glycolytic enzymes (10). Anaerobic glycolysis leads to hydrolysis of ATP (11) and to the accumulation of lactic acid, with a consequent decrease in pH. The average pH in a variety of tumors was found to be about 0.5 units lower than that of the surrounding normal tissues ([pH 6.5 to 6.9 and 7.0 to 7.5, respectively (12–15)], but pH values of 6.0 or lower have been recorded in tumors (16). Values of pH are likely to vary in different regions of tumors, and lower than average values would be expected to occur in areas of hypoxia.

Despite the limited capacity of cells to withstand acidification, the role of low pH in contributing to cell death in hypoxic regions of tumors has not been addressed. In a preliminary report (17) we showed that the combined effect of acidity and hypoxia could lead to a marked fall in the viability of cultured cells. The purpose of the present work was to extend these initial studies and to analyze characteristics of the interaction between hypoxia and an acidic environment which may lead to changes in cell survival and cellular energy metabolism.

Materials and Methods

Cells. CHO cells and the human bladder cancer cell line MGH-U1 (obtained from Dr. G. Prout, Urology Research Laboratory, Massachusetts General Hospital, Boston, MA) were maintained in complete a-medium (18) supplemented with antibiotics and 10% FCS. Cultures were reestablished from frozen stock at approximately 3-mo intervals. Both cell lines were free of Mycoplasma.

Prior to experiments, exponentially growing MGH-U1 cells were detached from their flasks using 0.025% trypsin and 0.01% EDTA, washed, and resuspended in fresh medium. CHO cells were maintained routinely in culture flasks and were transferred and grown in spinner culture for a few days prior to their use in experiments.

Cell Survival Experiments. A suspension of exponentially growing cells was washed and dialyzed in a-medium plus 5% DFCS to provide a concentration of 10^5 cells/mL. Volumes of 10 ml were added to small glass vials and stirred continuously at 37°C. A humidified gas mixture of 5% CO2 in air or nitrogen (<10 ppm O2) or in a specified intermediate oxygen concentration, flowed through the vials, as described previously (19). At appropriate times, 0.5 ml of the cell suspension was removed by passing the long needle of a syringe through the gas outlet. The cells were counted, and appropriate dilutions were plated in a-medium + 10% FCS in triplicate Petri dishes. Colonies were stained and counted 9 to 13 days later. In all experiments, acidic conditions were achieved by adding appropriate amounts of sodium bicarbonate (i.e., less than the usual concentration of 26 mm) to bicarbonate-free medium (± 5% DFCS) to obtain the desired pH. In experiments in
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Biochemical Assays. The concentration of L-lactate, pyruvate, and D-glucose in the incubation medium was measured with a spectrophotometer (Cary 219, Varian) using commercial kits (Sigma, St. Louis, MO). In the presence of excess NAD and lactate dehydrogenase, lactate is converted to pyruvate and NADH. The increase in absorbance at 340 nm due to the formation of NADH is directly proportional to lactate concentration. When the reverse reaction is carried out, the decrease of absorbance at 340 nm due to disappearance of NADH is proportional to pyruvate concentration. Determination of glucose levels is based on the conversion of glucose to glucose-6-phosphate by ATP in the presence of hexokinase, coupled with the subsequent reduction of NADP to NADPH; the absorbance of NADPH was measured at 340 nm. The initial glucose concentration in all experiments (unless otherwise stated) was 5.6 mM.

The cellular concentration of ATP, ADP, and AMP was measured by HPLC. The following chemicals were used. Acetonitrile and KH₂PO₄ (HPLC grade) were obtained from Fisher (Fair Lawn, NJ); TBAP from Waters Associates (Milford, MA); trichlorotrifluoroethane (Freon), from Matheson (Whitby, Ontario); and perchloric acid, from Baker (Phillipsburg, NJ). All other chemicals were purchased from Sigma (St. Louis, MO). For determination of adenine nucleotides, samples were prepared for analysis as described by Bump et al. (20). Briefly, 10⁶ cells were centrifuged (1000 rpm, for 5 min, at 4°C), washed once (for 5 min, including centrifugation time at 0°C) with 10 ml of ice-cold phosphate-buffered saline, deproteinized for 15 min in 1 ml of ice-cold perchloric acid (0.4 M), and sedimented, and the supernatant was stored at −70°C for up to 1 wk.

Perchloric acid was removed by extraction with 1 ml of 20% triethylamine in Freon, followed by four extractions with 1 ml of cold Freon. All extractions were carried out on ice. For preparation of standard solutions, increasing concentrations of AMP, ADP, and ATP dissolved in ice-cold PBS were added to 1 ml of ice-cold perchloric acid (0.4 M) for 15 min. Standard solutions were then treated in a manner identical to the experimental samples. For HPLC analyses, 5 to 10 μl of the extract (from the samples or the standard solutions) were injected into the HPLC instrument (Waters Associates, Milford, MA; Model 440 absorbance detector), using a reverse phase Nova Pak C₁₈ column with isocratic elution and a buffer containing 65 mM KH₂PO₄, 1 mM TBAP, and 5% acetonitrile at pH 3.2 (21). The flow rate was 1 ml/min, and absorbance was measured at 254 nm. The area under each peak was calculated by an integrator (Data Module 730; Waters Associates), and a standard curve was constructed from the peak area of the standards. A new set of standards was run for each experiment (each day), although all the standard curves obtained were similar. Peak identification was confirmed by the addition of a known quantity of AMP, ADP, and ATP (standard solution) to a duplicate sample, as shown in Fig. 5E. Energy charge was calculated as [(ATP + ½ ADP)/(AMP + ADP + ATP)] (22).

RESULTS

Effect of Hypoxia and Acidity on Cell Survival. Because of the different buffering capacity of media containing different concentrations of bicarbonate, medium pH was monitored over the 6-h duration of an experiment in which CHO cells (at 1 x 10⁶ cells/ml) were incubated at various initial values of pH and gassed with air or N₂ (each with 5% CO₂). The results show that medium pH increased following the addition of cells, but...
it decreased gradually after initiation of gassing, reaching values that were close to the initial medium pH (Fig. 1). The stability of medium pH over the course of 6-h incubation with cells was greater at lower pH (6.0 to 6.5) than at higher pH (~7.0) and was also greater under hypoxic conditions than in air.

Exposure of CHO cells to either hypoxia or low pH (in the range of pH 6.0 to 6.5) for up to 6-h had no effect on plating efficiency, but the combination of both conditions led to a marked decrease in cell survival. Loss of viability was dependent on duration of exposure (Fig. 2A) and on pH (Fig. 3A). For MGH-U1 cells (Fig. 2B), exposure to hypoxia alone for up to 6 h has no effect on viability, but exposure of aerobic cells at pH 6.0 was cytotoxic. The combination of hypoxia and acidic pH also resulted in a marked decrease in plating efficiency of MGH-U1 cells. Both types of cell showed no change in their ability to exclude trypan blue following 6-h incubation under any of the above conditions.

The oxygen sensitivity of pH-dependent cell killing of CHO cells is shown in Fig. 3B. At pH 6.2 ± 0.2 there was a rapid decline in plating efficiency as the oxygen concentration was decreased from about 0.2% (0.7 mm of Hg) to about 0.05% (0.2 mm of Hg).

Effect of Hypoxia and Acidity on Energy Metabolism. Experiments were undertaken to determine whether the observed decreases in cell survival could be correlated with changes in cellular energy metabolism. In both CHO and MGH-U1 cells, glucose consumption and lactate production were inhibited by low pH, with almost complete cessation of glycolysis at pH 6.0 (Fig. 4). Glucose consumption and lactate production by CHO cells were always higher under hypoxic conditions than in air (Fig. 4A), whereas they were similar for MGH-U1 cells under aerobic and hypoxic conditions (Fig. 4B). Net pyruvate removal from medium by both cell lines was not affected by pH and was slightly higher in air than in hypoxia (Fig. 4).

The influence of various conditions on levels of ATP and energy charge in CHO cells is summarized in Table 1. This table was constructed following HPLC analysis of levels of AMP, ADP, and ATP, as demonstrated in Fig. 5. Table 1 shows that, after 6-h incubation, energy charge was reduced below the normal value of 0.85 only in cells incubated under hypoxic conditions at low pH. The dependence of this fall in energy charge on time of incubation and on pH is shown in Fig. 6. The cellular concentration of ATP showed a larger
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Table 1. Relative concentrations of ATP and energy charge of CHO cells incubated for 6 h under the indicated conditions.

<table>
<thead>
<tr>
<th>Incubation condition</th>
<th>% of ATP*</th>
<th>Energy charge</th>
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<tbody>
<tr>
<td>A. Effect of hypoxia and low pH#</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Air, pH 7.0</td>
<td>100</td>
<td>0.85</td>
</tr>
<tr>
<td>N2, pH 7.0</td>
<td>115</td>
<td>0.88</td>
</tr>
<tr>
<td>Air, pH 6.0</td>
<td>99</td>
<td>0.87</td>
</tr>
<tr>
<td>N2, pH 6.0</td>
<td>14</td>
<td>0.64#</td>
</tr>
</tbody>
</table>

B. Effect of γ-irradiation#

- Control          100       | 0.86          |
- γ-irradiated (30 Gy) | 113       | 0.88          |

C. Effect of glucose depletion#

- N2, pH 7.0, -glucose | 2.5        | 0.56          |
- N2, pH 6.0, -glucose | 0.5        | 0.45          |

* ATP levels are expressed as percentage of controls, to account for any possible loss of ATP during preparation of cells prior to extraction with perchloric acid.

# Means of two to four experiments. All groups were incubated in the presence of glucose (5.6 mM). Multiple estimates of energy charge under control conditions are highly reproducible with a range of 0.82 to 0.88.

& Energy charge at the beginning of the experiments was 0.85.

$ Cells were incubated in standard α-medium plus 5% DFCS (pH 7.4).

| 5| 8
<table>
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<tbody>
<tr>
<td>A. 5μl of 25µM standard</td>
<td>0.025</td>
</tr>
<tr>
<td>B. 5μl of sample N2, PH 7.0, Glucose</td>
<td>0.000</td>
</tr>
<tr>
<td>C. 5μl of sample N2, PH 7.0, Glucose</td>
<td>0.005</td>
</tr>
<tr>
<td>D. 5μl of sample N2, PH 6.0, Glucose</td>
<td>0.000</td>
</tr>
<tr>
<td>E. 5μl mixture of 2/3 sample B and 1/3 5µM standard</td>
<td></td>
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</table>

Changes in energy metabolism of cells exposed to hypoxia and low pH might cause cell death or might simply be an early manifestation of loss of viability. We therefore studied the rate of glycolysis and energy charge following an independent method of causing cell death. A lethal dose of 30 Gy of γ-irradiation reduced the surviving fraction of CHO cells below 1.3 × 10^-4 but had no effect on their lactate production (Fig. 7) or energy charge (Table 1B) for at least 6 h.

Effect of Glucose and Pyruvate on Survival and Energy Metabolism of CHO Cells. To investigate further whether changes in energy metabolism might be causally related to cell death, we incubated cells in a hypoxic environment in the absence of glucose. We therefore removed the major substrate for glycolysis and prevented oxidative phosphorylation. As expected, these conditions led to almost total inhibition of lactate production (Fig. 8) and to a fall in energy charge to an even lower value than that observed under hypoxic conditions at pH 6.0 (Fig. 5; Table 1C). However, cell survival fell only to about 10^-2 when cells were incubated for 6 h under hypoxic conditions at pH 7.0 in the absence of glucose (Fig. 9); this is a much higher surviving fraction than was observed when the cells were incubated at pH 6.0 under hypoxic conditions in the presence of glucose (5.6 mM).

Removal of glucose from the medium also led to a lower cell survival under hypoxic conditions at pH 6.0 (Fig. 9) than in the presence of glucose. Removal of pyruvate had a similar (albeit smaller) effect to lower survival of hypoxic cells at pH 6.0, but it had no effect at pH 6.5 or 7.0 (data not shown). The low...
Fig. 7. Lactate production by CHO cells at intervals after 30-Gy γ-irradiation and by unirradiated control cells. Data represent values for three measurements of lactate concentration in cell-free medium following incubation of 1×10⁶ cells/ml at pH 7.4. The experiment was repeated, and similar results obtained.

production of lactate under hypoxic conditions at pH 6.0 was further inhibited by removal of glucose (Fig. 8) or pyruvate (data not shown) from the medium.

DISCUSSION

The present experiments have demonstrated a marked decrease in survival of two types of cells when they were incubated under hypoxic conditions at low pH. Hypoxia and an acidic environment are known to occur in regions of solid tumors, and our results suggest that these conditions may contribute to the process of natural cell death which occurs in solid tumors. Lethal effects are observed in the range of pH 6.0 to 6.5, and although this range is lower than average measurements of pH in most solid tumors (12-16), regional hypoxia with consequent production of lactic acid is likely to lead to tumor regions with values of pH in this range. Oxygen sensitivity of the lethal effect of low pH occurs over a similar range of pO₂ to that which influences radiation sensitivity, although it seems unlikely that these effects occur through related mechanisms.

Fig. 8. Lactate production by CHO cells incubated with or without 5.6 mM glucose (glc), under air (closed symbols) or N₂ (open symbols) at pH 7.0 or 6.0. Values represent mean lactate concentration of three measurements carried out on cell-free media following incubation of 1×10⁶ cells/ml for up to 6 h. • and ○, pH 7.0, with glucose; □ and △, pH 7.0, without glucose; ▼ and □, pH 6.0 with glucose; △ and △, pH 6.0, without glucose.

Fig. 9. Plating efficiency of CHO cells assessed in an experiment identical to that described in Fig. 8. Symbols are as in Fig. 8. Data represent mean and range of values for triplicate plates. Similar results were obtained in two replicate experiments.
In both CHO and MGH-U1 cells, glucose consumption and lactate production were suppressed as the pH of the medium was lowered from 7.0 to 6.0, confirming earlier reports that glycolysis is inhibited at low pH (23). This effect is probably due to inactivation of phosphofructokinase, the main regulatory enzyme in glycolysis, at low pH (24–26). Thus, one possible explanation for the cell death observed under conditions of hypoxia and acidity is that cells incubated in hypoxia (a condition in which respiration is inhibited) and low pH (a condition in which glycolysis is inhibited) might die of energy deprivation. The observed reduction in ATP levels and in energy charge of CHO cells under these conditions supports the view that energy deprivation might contribute to cell death, especially since the range of pH sensitivity (6.5 to 6.0) is similar for the decrease of both energy charge and cell survival. Although the levels of phosphocreatine were not measured, this compound was not likely to be a major source of energy in our cells, which were not of muscle or nerve origin.

The absence of an enhanced rate of glycolysis under hypoxic conditions for MGH-U1 cells is consistent with earlier studies (e.g., Ref. 27) which show that many tumor cells utilize glycolysis even in the presence of oxygen. CHO cells, which are not malignant, show the normal increase in rate of glycolysis under hypoxic conditions relative to air.

CHO cells which received a lethal dose of X-rays had a normal rate of glycolysis for 24 h and normal energy charge for at least 6 h after irradiation. Cells that were lethally damaged by exposure to hypoxia and low pH maintained intact membranes and excluded dye during the 6-h period of incubation, similar to those treated with radiation. It seems unlikely therefore that the changes in metabolism observed within 6 h of incubation under hypoxia and low pH were a nonspecific result of cell death, although we cannot exclude this possibility with certainty.

In agreement with earlier studies (28, 29), we have found that inhibition of glycolysis by the strategy of removing glucose and oxygen led to a decrease of cell survival and reduction of ATP levels and energy charge. In our experiments, this reduction in cell survival was much smaller than that observed following incubation of cells at pH 6.0 (with glucose) under hypoxia. This smaller cell kill could not be attributed to continuing glycolysis from breakdown of glycogen stores, because the rate of lactate production under hypoxic conditions was similar (and very low; Fig. 8) in cells incubated without glucose and in cells incubated with glucose at pH 6.0. The observation that cell survival was higher but energy charge was lower (Table 1; Fig. 9) in hypoxic cells incubated in the absence of glucose (at pH 7.0) than in hypoxic cells incubated at pH 6.0 (with glucose) suggests that mechanisms other than energy deprivation were contributing to cell death. It is possible that hypoxia inhibited recovery from intracellular acidification in cells placed in an acidic environment, as shown for Ehrlich ascites tumor cells (28, 30). Such recovery appears to depend on ion exchange pumps (e.g., Na+/H+ and/or HCO3−/Cl−) located in the cell membrane. We are currently investigating the effect on cell viability of agents which enhance intracellular acidification by interference with the normal function of these ion exchange pumps.

Because cells in nutrient-deficient regions of tumors are known to be resistant to radiation and to some of the conventionally used anticancer drugs (31–33), imitation or stimulation of mechanisms which lead to natural tumor cell death might have considerable potential in tumor therapy. Results of the present study may aid in the selection and development of drugs with activity against nutritionally deprived tumor cells.

REFERENCES


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