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ORIGINAL ARTICLE

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Greater cell cycle inhibition and cytotoxicity induced by 2-deoxy-D-glucose in tumor cells treated under hypoxic vs aerobic conditions

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Abstract *Purpose*: In order to investigate the hypothesis that cells found in hypoxic areas of solid tumors are more sensitive to glycolytic inhibitors than cells growing aerobically, we have previously characterized three distinct in vitro models (A, B and C) that simulate this condition. In all of the models it was shown that cells growing under hypoxic conditions are hypersensitive to the glycolytic inhibitor 2-deoxy-D-glucose (2-DG). However, in those studies cytostatic and cytotoxic effects were not distinguished from one another. Since successful treatment of cancer includes not only slowing down but also actually killing tumor cells, studies were undertaken to assess the effects of 2-DG on cell cycle progression and cell death. *Methods and results*: Using flow cytometry and cell viability assays, it was found that 2-DG caused significantly greater cell cycle inhibition and cell death in all three hypoxic models as compared to aerobically growing control cells. In model A (a chemically induced model of hypoxia in which rhodamine-123 is used to block oxidative phosphorylation), 1200 μg/ml of 2-DG was shown to induce more cell cycle arrest in late S/G_2 and more cell death than in the aerobic cell counterpart treated with 3600 μg/ml 2-DG. In ρ^0 cells which are genetically constructed to be unable to perform oxidative phosphorylation (model B), an even greater window of selectivity (more than tenfold) between hypoxic and aerobic cells was found when considering 2-DG's effects on cell cycle arrest and cell death. In the environmental model (model C), where cells were grown under reduced amounts of external oxygen (0.1%), hypersensitivity to the effects of 2-DG with respect to cell cycle arrest and cell death were also observed. *Conclusions*: Overall, these results indicate that cells growing under anaerobic conditions respond with greater sensitivity to the effects of 2-DG on cell cycle inhibition and cell death than those growing under aerobic conditions. This supports our contention that glycolytic inhibitors added to standard chemotherapeutic protocols should increase treatment efficacy by selectively killing the slow-growing cells, which are found in the hypoxic portions of solid tumors, while sparing most of the normal cells that are also slow-growing but are living under aerobic conditions.

Keywords Hypoxia · 2-Deoxy-D-glucose · Glycolysis · Oxidative phosphorylation · Osteosarcoma

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Introduction

Since most of the cancer chemotherapeutic agents used in the clinic are toxic to rapidly dividing cells, the slow-growing cells generally found in the hypoxic areas of solid tumors remain relatively unaffected. However, under hypoxic conditions, cells switch from aerobic to anaerobic metabolism. This switch offers the possibility to selectively target slow-growing tumor cells by using inhibitors of glycolysis, since hypoxic cells must rely solely on this metabolic pathway for ATP production. In contrast, aerobically metabolizing cells can use carbon sources other than glucose (i.e. fatty acids and amino acids) to produce ATP via oxidative phosphorylation (OxPhos) [3, 7, 11, 18, 20, 22], and therefore should be able to withstand blockage of glycolysis. Conversely, a hypoxic cell treated with a glycolytic inhibitor should be more severely affected, since without oxygen it cannot use these alternative carbon sources to generate ATP. Thus, a window of selectively should exist between aerobically growing cells found in most human tissues

and slow-growing cells found in the hypoxic portions of most solid tumors, which can be exploited for therapy by using inhibitors of glycolysis.

To investigate this premise, three distinct in vitro models of "hypoxia" (models A, B and C) were developed and characterized [6, 13, 14]. Chemical model A approximates hypoxia by using agents such as rhodamine-123 (Rho123), rotenone, antimycin A, and oligomycin, which interfere with mitochondrial OxPhos. Genetic model B uses osteosarcoma cells (ρ^0) that have lost their mitochondrial DNA and therefore cannot undergo OxPhos. Environmental model C is represented by tumor cells grown under decreased levels of atmospheric oxygen [14]. Note that the term hypoxia in this communication is put in quotes to indicate that models A and B are not truly hypoxic since they are growing under normoxic conditions. However, they both share in common an inability to undergo OxPhos, in essence simulating hypoxia. Model C more closely resembles the in vivo conditions of hypoxia.

Previous results have shown that cells in all of the models are more sensitive to inhibitors of glycolysis, i.e. 2-deoxy-D-glucose (2-DG) and oxamate, than cells growing under aerobic conditions [6, 13, 14]. Moreover, the "hypoxic" cells in these models produce increased levels of lactic acid, indicating that they are indeed metabolizing anaerobically [13, 14]. Our results, however, did not measure the effects of glycolytic inhibitors on cell cycle progression, nor did they distinguish between cytostatic and cytotoxic effects in tumor cells growing under aerobic or anaerobic conditions. Since the success of using glycolytic inhibitors for clinical treatment lies in their potential to actually kill slow-growing hypoxic tumor cells, while sparing the slow-growing aerobically metabolizing normal cells, here we used laser flow cytometry and viable counts to investigate the effects of 2-DG on cell cycle progression and cell death in the three models of "hypoxia."

Materials and methods

Cell types

An osteosarcoma cell line 143b (wild-type, wt) was exposed to ethidium bromide for prolonged periods and a mutant cell line with complete loss of mtDNA (ρ^0) was selected [9]. Since the ρ^0 cells are uridine and pyruvate auxotrophs they were grown in DMEM supplemented with 10% fetal calf serum, 50 μg/ml of uridine, 100 m*M* sodium pyruvate and 10 μg/ml of gentamicin. To maintain standard experimental conditions the parental cell line (wt) was grown in the same medium.

Drugs

Rho123 and 2-DG were obtained from Sigma (St. Louis, Mo.).

Direct cytotoxicity assays

For direct cytotoxicity assays, 2 ml of 143b and ρ^0 cells were seeded in 24-well plates at 3×10^4 and 5×10^4 cells per well, respectively. Cells were incubated for 24 h at 37°C in an atmosphere containing 5% CO_2. Drug treatments were then applied and were continuous for 72 h. Cells and their respective culture medium were collected, combined, and centrifuged at 400 *g* for 5 min. Cell pellets were resuspended in 1 ml fresh culture medium and 0.5 ml trypan blue (as an indicator of cell death). Trypan blue-positive cells were scored using a hemocytometer, and the percentage of dead cells was calculated.

Hypoxia

For studies on glycolytic inhibitors in model C, cells were seeded and incubated for 24 h at 37°C in an atmosphere containing 5% CO_2 as described above (direct cytotoxicity assays). After a 24-h incubation, cells received drug treatment and were placed in a Pro-Ox in vitro chamber attached to a model 110 oxygen controller (Reming Bioinstruments, Redfield, N.Y.). A mixture of 95% nitrogen and 5% CO_2 was used to perfuse the chamber to achieve the desired oxygen level (0.1%). Hypoxic treatment was continuous for 72 h.

Rapid DNA content analysis

Cells were cultured, treated, and pelleted as described above for the direct cytotoxicity assays. Cell pellets were resuspended in 1.5 ml propidium iodide/hypotonic citrate staining solution [10]. Stained cells were analyzed in a Coulter XL flow cytometer to determine nuclear DNA content and cell cycle position. A minimum of 10,000 cells were analyzed to generate a DNA distribution histogram.

Results

Chemical model A: effects of 2-DG on cell cycle progression and death

A number of mitochondrial inhibitors including Rho123 have been previously found to hypersensitize osteosarcoma cells to glycolytic inhibitors [13]. Here, laser flow cytometry for analysis of DNA content and cell cycle traverse demonstrated that when cells were treated simultaneously with Rho123 and 2-DG for 72 h, they arrested in late S/G_2 and died. Moreover, these responses were more pronounced with increasing doses of 2-DG (Fig. 1). The DNA distribution histograms shown in Fig. 1A contain two prominent peaks, representing cells in G_1 (left) and late $S/G_2/M$ (right) with S phase cells lying between these peaks. When cells were treated with both Rho123 (1.5 μg/ml) and 2-DG (1200 μg/ml), a peak with less than G_0/G_1 DNA content appeared, which represented dead cells having undergone necrosis and/or apoptosis. In addition, there was significant accumulation of cells in late S/G_2. These effects on cell cycle and cell death were more intense at a higher dose of 2-DG (3600 μg/ml). In contrast, 2-DG alone at 3600 μg/ml produced little or no effect on cell cycle traverse and only a small increase in cells with DNA content less than G_0/G_1. Similarly, only a slight accumulation of cells in late S/G_2 and no increase in cell death occurred when cells were treated with Rho123 alone at 1.5 μg/ml. Thus, it is clear that under chemical "hypoxia" (model A), blocking glycolysis resulted in both arrest of cells in late S/G_2 and cell death.

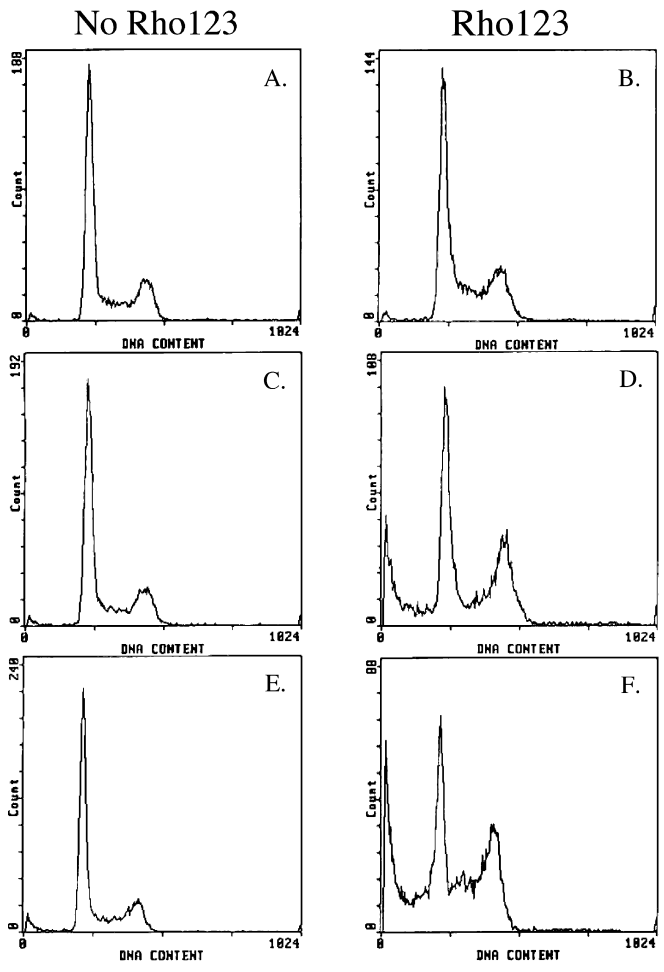

Fig. 1A–F DNA distribution histograms of 143b osteosarcoma cells exposed to 2-DG in the presence (model A) and absence of Rho123 (1.5 μg/ml). **A** Untreated control culture; **B** cells treated with Rho123 alone; **C, E** cells cultured without Rho123 and treated with 1200 and 3600 μg/ml 2-DG, respectively; **D, F** cells treated with a combination of Rho123 and 1200 and 3600 μg/ml 2-DG, respectively. Note that in the Rho123-treated cultures (**B**, **D** and **F**) the pre-G_1 as well as the late S/G_2 peaks are elevated with increasing doses of 2-DG while the untreated (without Rho123) cultures remain relatively unaffected at equivalent doses of 2-DG

Genetic model B: cytotoxic and cell cycle effects of 2-DG in ρ^0 cells

In this genetic model of hypoxia, 2-DG treatment of ρ^0 cells elicited cytotoxic and cell cycle blocking effects similar to those obtained in model A, albeit somewhat more pronounced (Fig. 2). At 300 μg/ml of 2-DG, these cells showed an increased profile of DNA content less than G_0/G_1 (indicating cell death) and a slight increase in accumulation in late S/G_2. These effects increased with each subsequent dose. In contrast, in wt (aerobic) cells, only a small amount of cytotoxicity was seen at the highest dose applied (3600 μg/ml). Thus, 2-DG induced more severe effects on cell cycle traverse and cell viability at 300 μg/ml in the genetically anaerobic ρ^0 cells than 3600 μg/ml in the aerobic metabolizing wt cells.

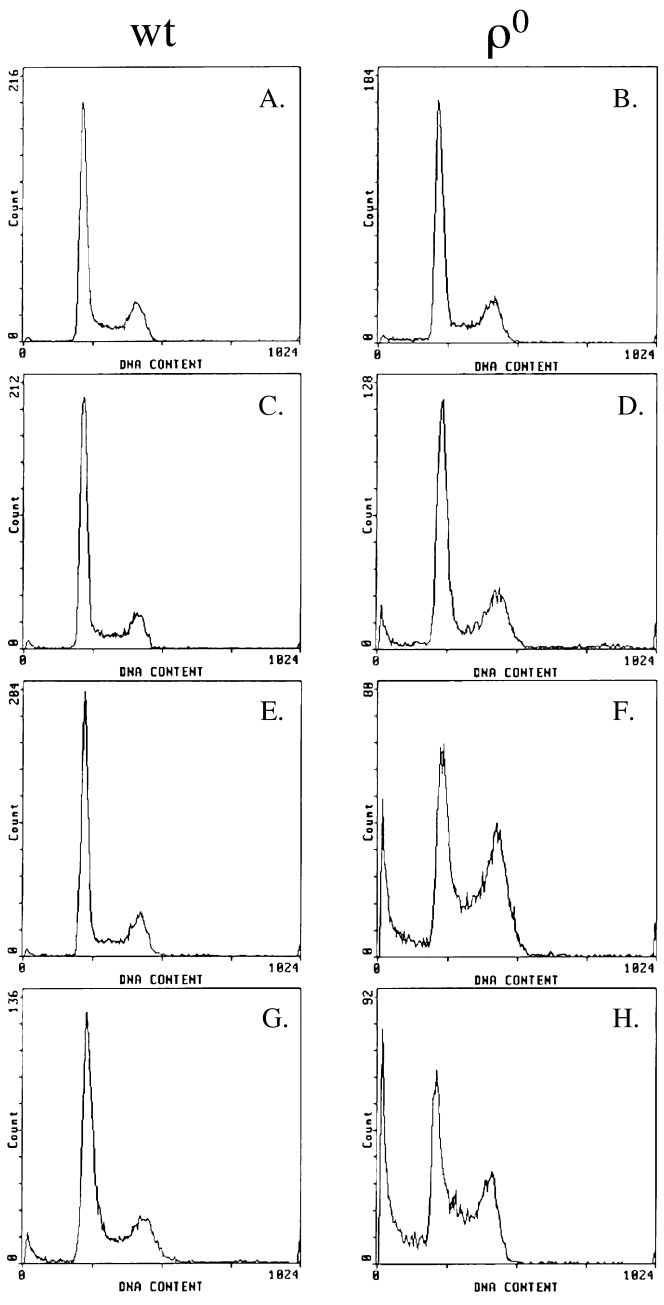

Fig. 2A–H DNA distribution histograms of wt (143b) and ρ^0 (206) osteosarcoma cells (model B) after exposure to 2-DG. **A** Untreated wt control culture; **B** untreated ρ^0 control culture; **C, E, G** wt cells treated with 300, 1200 and 3600 μg/ml 2-DG, respectively; **D, F, H** ρ^0 cells treated with 300, 1200 and 3600 μg/ml 2-DG, respectively. Note the increased sensitivity indicated by the rise in the pre-G_1 and the late S/G_2 peaks of the ρ^0 cells (**B**, **D**, **F** and **H**) as compared to the relatively minor effects on the wt cell counterparts at equivalent 2-DG doses (**A**, **C**, **E**, and **G**)

Environmental model C: effects of 2-DG on the cell cycle and viability of hypoxic cells

To assess the effects of 2-DG in an in vitro model more closely resembling tumor hypoxia in vivo and to compare them to those found in models A and B, wt cells were cultured under hypoxic conditions, achieved by

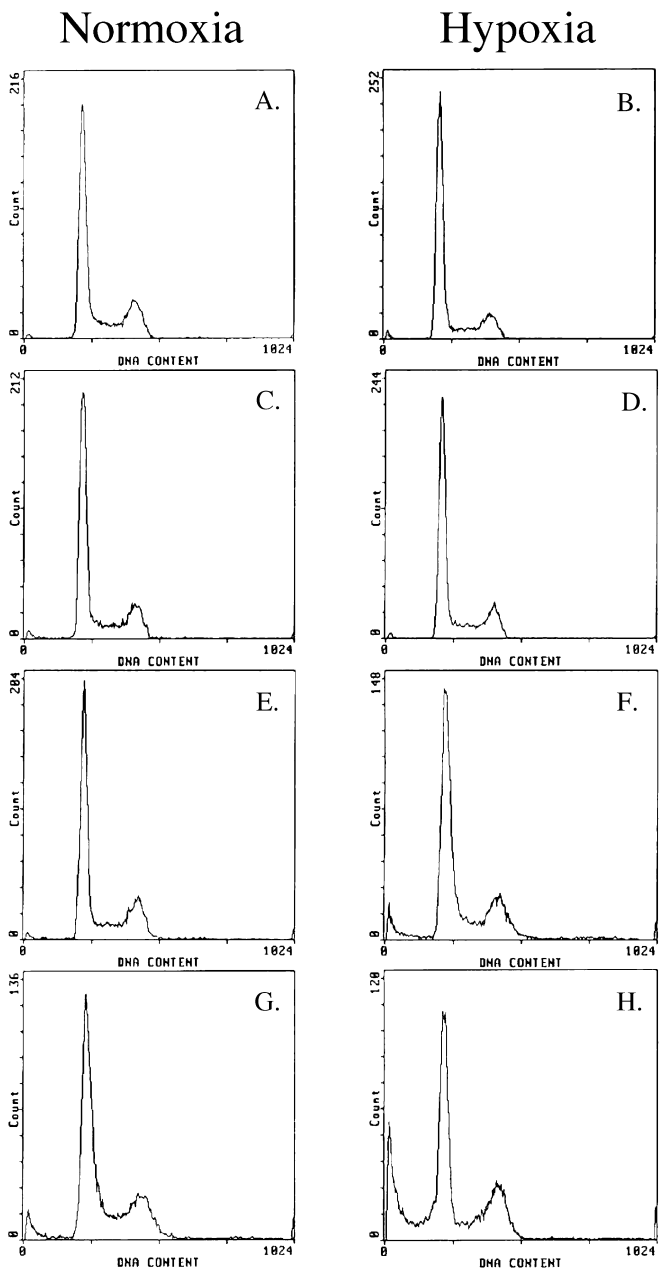

Fig. 3A–H DNA distribution histograms of 143b osteosarcoma cells after exposure to hypoxic (0.1% O_2; model C) or normoxic (21% O_2) culture conditions and 2-DG treatment. **A** Untreated normoxic (21% external O_2) control culture; **B** untreated hypoxic (0.1% external O_2) control culture; **C, E, G** cells under normoxic conditions treated with 300, 1200 and 3600 μg/ml 2-DG, respectively; **D, F, H** hypoxic cells treated with 300, 1200 and 3600 μg/ml 2-DG, respectively. Note that hypoxia increases the effects of 2-DG treatment

lowering external oxygen to 0.1%, and treated with various doses of 2-DG. In cultures exposed to 1200 μg/ml of 2-DG cell death and cell cycle block in late S/G_2 occurred as demonstrated by the DNA distribution histogram in Fig. 3. These effects were increased in cultures treated with 2-DG at the higher dose of 3600 μg/ml (Fig. 3). However, cells grown under aerobic conditions treated with 2-DG at the highest dose used in these experiments showed only minor effects on cell cycle traverse and viability. Although less pronounced as compared to the chemical and genetic models, it is clear from these results that a hypoxic environment of 0.1% external oxygen increased the cytotoxic and cell cycle effects of 2-DG treatment in these cells.

Trypan blue assays of cell death after 2-DG treatment in all three "hypoxic" models

In order to more directly measure cell death and to confirm the data from models A, B and C, cell viability experiments were performed. For each model, cells and culture medium were collected and combined, and trypan blue was added to the mixture. Trypan blue-positive cells were scored and the percentage of dead cells was calculated.

In the absence of 2-DG, the wt aerobically growing control cells and the cells of the three "hypoxic" models displayed the same low amount (< 10%) of cell death (Fig. 4) when cultured for 3 days. When 2-DG was applied to the wt control cultures at either 1200 or 3600 μg/ml, cell death remained below 10% at the lower dose and increased to 17.31% at the higher dose. In contrast, in all three "hypoxic" models, cell death was found to be significantly higher at both 2-DG doses. The percentage of dead cells at 1200 and 3600 μg/ml of 2-DG was 37.8% and 74.5% in model A, 50.8% and 74.0% in model B and 21.2% and 54.4% in model C, respectively. Thus, the data in Fig. 4 support the flow cytometry results which indicate that cells under "hypoxia" (models A, B and C) were killed more effectively with 2-DG treatment than cells growing aerobically.

Discussion

The data obtained here with the three models of simulated hypoxia illustrate that when cells growing anaerobically were treated with an inhibitor of glycolysis, they underwent cell cycle arrest and cell death. This is in marked contrast to what was found for aerobically metabolizing cells, which were able withstand high levels of 2-DG with relatively little cell cycle interruption or cell death. The degree of the effects of 2-DG on the cell cycle and viability in anaerobically growing cells, however, varied according to the model treated, which was not unexpected since each condition of "hypoxia" was different.

In the chemical model an increased accumulation of cells in late S/G_2 and a greater amount of cell death was observed with escalating 2-DG concentrations (1200 and 3600 μg/ml) when assayed by flow cytometry (Fig. 1). However, control cells growing in the absence of Rho123 showed little or no effect of 2-DG at either dose. Thus, a greater than threefold window of selectivity existed between the chemically induced anaerobic and

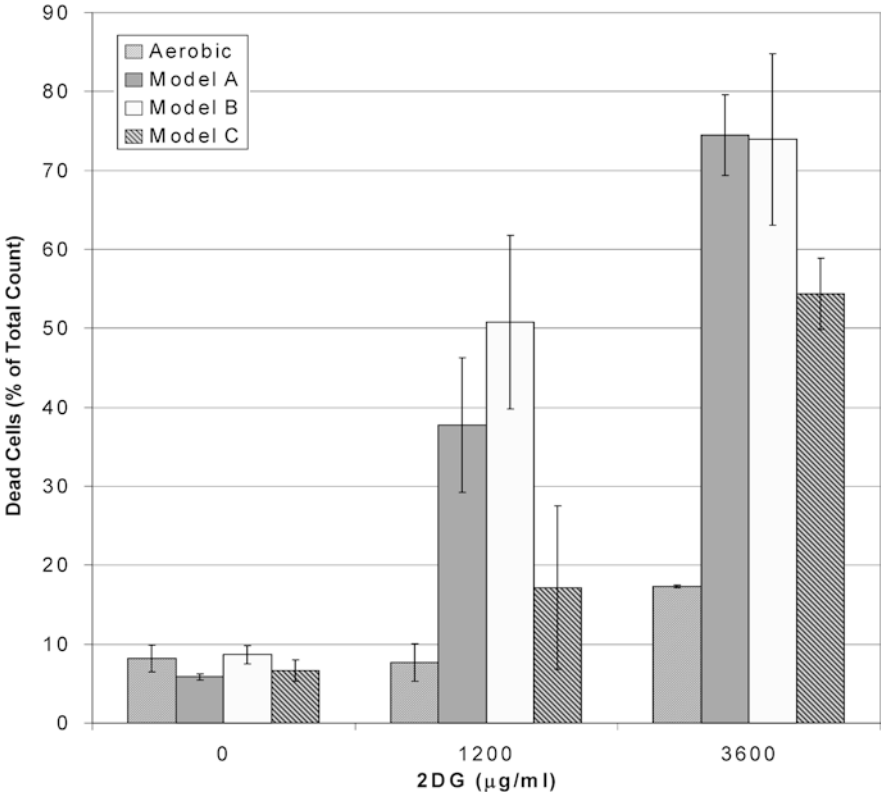

Fig. 4 Direct cytotoxicity assays of the "hypoxic" cell models (A, B and C) after exposure to various doses of 2-DG. Note the increase in cell death after 2-DG treatment in each model when compared to the aerobic control. Cell death was calculated as a the percentage of trypan blue-positive cells in the culture

aerobic metabolizing cells when treated with 2-DG. Similarly, as shown in direct cytotoxicity assays, in which both attached cells and their respective culture mediums were collected and trypan blue was used as an indicator of cell death, 2-DG produced a four- to five-fold greater effect in anaerobically growing cells than in aerobically growing cells.

In genetic model B, this window between anaerobic and aerobic cells was greater than in model A. Thus, the effects on cell cycle traverse and cell death seen at a dose of 300 μg/ml 2-DG in the anaerobic cells were greater than those observed in aerobically growing cells treated with a tenfold higher dose (3600 μg/ml). This larger window of selectivity in the genetic model was also seen when the percentage of dead cells was assayed directly using trypan blue (Fig. 4). The most likely explanation to account for the greater potency of 2-DG in model B than in model A appears to be that OxPhos was completely absent in the former but perhaps not completely shut down at the Rho123 doses used in the latter. In fact it has been shown that in isolated mitochondria, 1.5 μg/ml of Rho123 reduces the respiration control ratio and ATP synthesis by less than 50 indicating that mitochondria still function at this dose [12, 19]. Although extrapolating the amount of drug necessary to inhibit isolated mitochondria to that required to completely block mitochondria in situ requires consideration of drug transport through the plasma as well as the mitochondrial membrane, overall the data support the idea that Rho123 may not have been at a high enough dose in the present experiments to completely inhibit OxPhos.

In the environmental model of hypoxia (model C) the effects of 2-DG were similar but less than those found in models A and B. This diminished effect may be explained not only by OxPhos still partially functioning at the levels of oxygen used in model C (0.1%), but also by increases in hypoxia-inducible factor (HIF) [24] that have been reported for the same cell line exposed to hypoxia [23]. Since HIF is known to act as a transcription factor for genes involved in glucose transport and glycolysis [15, 17, 21, 24], it is likely that 2-DG uptake and activity is affected by the expression of these genes. On the one hand, 2-DG uptake should be enhanced by increases in glut transporters and thus cells should become more sensitive since it has been shown that 2-DG uses the same transporters as glucose [1, 4, 8]. On the other hand, hexokinase, the enzyme used to convert 2-DG to 2-deoxyglucose-6-phosphate is also upregulated by HIF [17], and therefore the cell may become more resistant since there is more enzyme to be inhibited by a given amount of 2-DG. It is thus difficult to predict a priori how HIF produced in response to hypoxia will affect sensitivity to 2-DG or other inhibitors of glycolysis. However, in models A and B, HIF does not appear to be a factor [23] which suggests that HIF induction in model C may be contributing to lowered sensitivity to 2-DG treatment. In regard to this latter point, using a mutant cell line which is unable to initiate an HIF response [5, 16, 25], we found that under hypoxia this cell line was more sensitive to treatment with 2-DG than its wild-type HIF-positive counterpart (data not shown). These in vitro results provide direct evidence to show

that HIF mediates resistance to glycolytic inhibitors which may be, at least in part, due to upregulation of the glycolytic enzymes. HIF also induces the expression of vascular endothelial growth factor (VEGF) [17], thereby increasing the vasculature of the tumor in vivo. Therefore, inhibiting HIF should theoretically increase the effectiveness of 2-DG treatment in the clinic by suppressing the induction of glycolytic enzymes and/or increasing hypoxia via blockage of angiogenesis.

Several reports have indicated that not only is the breakdown of glucose important for the energy requirements of a cell, but that the glycolytic intermediates, i.e. glucose-6-phosphate, fructose-6-phosphate and triosephosphate, also act as carbon sources for the ribose-5-phosphate skeleton required for nucleotide pools [3, 7, 18, 20]. In addition, these glycolytic intermediates are known to be precursors for various macromolecules including non-essential amino acids and glycerol [18, 20]. It has been suggested that there is ongoing competition for the carbons of glucose between the cell's requirements for energy and its needs for RNA, DNA and macromolecule synthesis [7, 18, 20]. This competition could explain why the "hypoxic" cells in our models were more affected by inhibitors of glycolysis than the aerobic cells (see summary illustration Fig. 5). In the case of the aerobic cell, when glycolysis is inhibited by 2-DG, carbon sources other than glucose such as amino acids or fatty acids, can be used for energy production via OxPhos [3, 7, 11, 18, 20, 22]. However, in a hypoxic cell, since mitochondrial OxPhos is non-functional, energy cannot be derived from any of these other carbon sources. Thus, when glycolysis is blocked with 2-DG, nucleic acid, macromolecule and ATP synthesis are compromised, leading to cell cycle arrest and cell death. The pile-up of hypoxic cells in S and G_2 at 2-DG doses significantly lower than those required to affect aerobic cells, could be a consequence of the former simply running out of energy and macromolecules necessary for cell cycle traverse and maintaining viability. In contrast, aerobic cells are better able to survive glycolytic inhibition by using alternative carbon sources for ATP production, thereby freeing the glycolytic intermediates for RNA, DNA and macromolecule synthesis. In support of this, we found that addition of 4 m*M* glutamine to cells growing aerobically treated with 1000 μg/ml of 2-DG showed a 100% increase in growth whereas ρ^0 cells similarly treated showed little ($< 20\%$) increase in growth (data not shown). More extensive

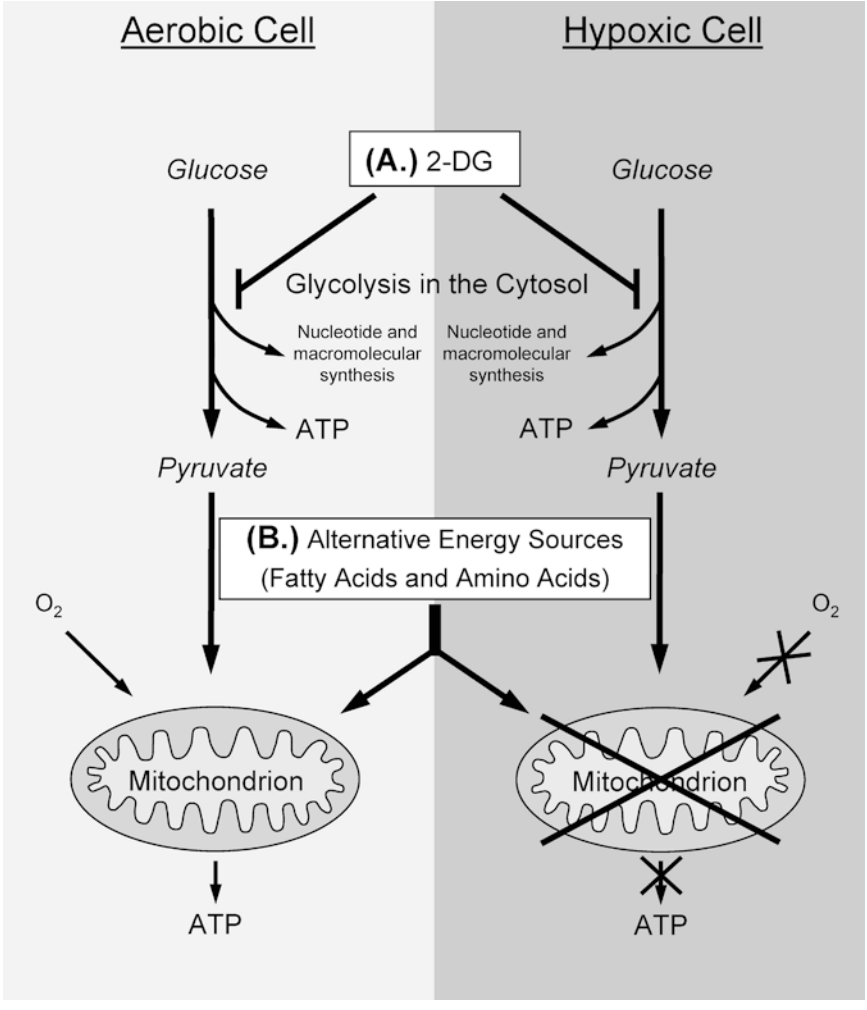

Fig. 5 Schematic illustration demonstrating different consequences of blocking glycolysis in aerobic vs hypoxic cells. In the aerobic cell, if glycolysis is inhibited by 2-DG (*A*), ATP cannot be generated by this pathway. However, since O_2 is available to the mitochondria, amino and/or fatty acids (*B*) can act as energy-providing carbon sources for OxPhos to take place, producing ATP. In contrast, when glycolysis is blocked in the hypoxic cell other carbon sources cannot be used by mitochondria since O_2 is unavailable and consequently OxPhos cannot take place. Thus, when glycolysis is blocked in the hypoxic cell, it has no alternative means for generating ATP and therefore will eventually succumb to this treatment

studies are ongoing to determine whether carbon sources other than sugars can rescue aerobically but not anaerobically metabolizing cells from glycolytic inhibition.

Previously, it has been shown that certain multidrug resistant (MDR) cell lines that are particularly sensitive to 2-DG, die primarily by apoptosis when treated with this glycolytic inhibitor alone [2]. In those studies it was suggested that hypersensitivity could be due to certain changes which occurred in these MDR cell lines as a consequence of the agent used to select for MDR mutants. Regardless of the reason for hypersensitivity to 2-DG in these MDR cell lines, it will be interesting to determine the nature of cell death (apoptosis and/or necrosis) in cells exposed to glycolytic inhibitors in our three models of hypoxia. These studies are the focus of future experiments.

The overall goal of our in vitro studies is to provide enough data to stimulate interest in developing and applying the concept of using glycolytic inhibitors to enhance the activity of current chemotherapeutic protocols, which are geared at targeting the rapidly dividing cells of a tumor. Thus, from the data derived here it is anticipated that addition of glycolytic inhibitors to standard cancer treatment regiments would increase their efficacy by selectively producing a cytotoxic effect on the slow-growing population of hypoxic tumor cells. Since anti-HIF as well as antiangiogenic therapies should create a more hypoxic microenvironment in solid tumors, the addition of 2-DG to these treatments could be anticipated to increase their efficacy.

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