Sensitization of Glioblastoma Cells to Irradiation by Modulating the Glucose Metabolism

Han Shen¹, Eric Hau¹,², Swapna Joshi¹, Pierre J. Dilda³, and Kerrie L. McDonald¹

Abstract

Because radiotherapy significantly increases median survival in patients with glioblastoma, the modulation of radiation resistance is of significant interest. High glycolytic states of tumor cells are known to correlate strongly with radioresistance; thus, the concept of metabolic targeting needs to be investigated in combination with radiotherapy. Metabolically, the elevated glycolysis in glioblastoma cells was observed postradiotherapy together with upregulated hypoxia-inducible factor (HIF)-1a and its target pyruvate dehydrogenase kinase 1 (PDK1). Dichloroacetate, a PDK inhibitor currently being used to treat lactic acidosis, can modify tumor metabolism by activating mitochondrial activity to force glycolytic tumor cells into oxidative phosphorylation. Dichloroacetate alone demonstrated modest antitumor effects in both in vitro and in vivo models of glioblastoma and has the ability to reverse the radiotherapy-induced glycolytic shift when given in combination. In vitro, an enhanced inhibition of clonogenicity of a panel of glioblastoma cells was observed when dichloroacetate was combined with radiotherapy. Further mechanistic investigations revealed that dichloroacetate sensitized glioblastoma cells to radiotherapy by inducing the cell-cycle arrest at the G2–M phase, reducing mitochondrial reserve capacity, and increasing the oxidative stress as well as DNA damage in glioblastoma cells together with radiotherapy. In vivo, the combinatorial treatment of dichloroacetate and radiotherapy improved the survival of orthotopic glioblastoma-bearing mice. In conclusion, this study provides the proof of concept that dichloroacetate can effectively sensitize glioblastoma cells to radiotherapy by modulating the metabolic state of tumor cells. These findings warrant further evaluation of the combination of dichloroacetate and radiotherapy in clinical trials.

Introduction

Glioblastoma is the most malignant form of primary brain tumor in adults. Despite increasing attention on targeted therapeutics in the treatment of glioblastoma, radiotherapy remains the most clinically effective treatment modality (1). However, radiotherapy only offers palliation, the efficacy of which is often limited by the occurrence of radioresistance, reflected as a diminished susceptibility of the irradiated cells to undergo cell death (2). To enhance the tumor cell sensitivity to radiotherapy, the mechanisms underlying radioresistance need to be further elucidated and strategies developed to overcome them.

When ionizing radiation passes the living tissue, the ionization of H2O leads to the production of reactive oxygen species (ROS) that contain chemically active oxygen molecules leading to oxidative stress and DNA damage. Oxygen molecules (O2) can stabilize the chemical composition of the DNA damage by reacting with the free radicals, such that O2 chemically “fixes” DNA damage. Unlike the balance achieved in normal tissues, the consumption of O2 by tumor tissue is much higher than the O2 supply from the surrounding blood vessels. Malignant solid tumors with inadequate blood supply and inconsistent perfusion therefore contain large portions of hypoxic cells which exhibit a high degree of resistance to chemoradiotherapy due, in part, to an increase of hypoxia-inducible factor-1α (HIF1α) and expression of other cellular survival molecules (3). Radiation itself, has been shown to stabilize the activity of HIF1α, which in turn regulates a plethora of genes involved in angiogenesis, invasion, metabolism, and protection against oxidative stress (4). The residual tumor cells surviving after chemoradiotherapy eventually proliferate and lead to cancer relapse.

It has long been known that the metabolism of solid tumors is radically different from that in the corresponding normal tissues. Numerous studies have demonstrated that tumor cells predominantly utilize glycolysis even in the presence of ample oxygen, referred as the Warburg effect. Using glycolysis provides a growth advantage for tumor cells and leads to malignant progression (5). Glioblastoma, like most malignant solid tumors, is highly glycolytic, producing large amounts of lactic acid as a metabolic byproduct. It has been shown that tumors with high levels of glycolysis are less responsive to radiotherapy and behave more aggressively (6). More recent reports have identified the Warburg effect to be implicated in resistance to cytotoxic stress induced by either chemotherapy or radiotherapy (7). In this way, treatment methods which block or reduce glycolytic metabolism may increase tumor cell sensitivity to radiotherapy.

Under hypoxic conditions, HIF1α causes an increase in its target gene pyruvate dehydrogenase kinase 1 (PDK1), which acts to limit the amount of pyruvate entering the citric acid cycle,
leading to decreased mitochondrial oxygen consumption (8). These findings suggest that inhibition of PDK1 could alter the glucose metabolism and increase oxygen consumption of tumor cells, which would sensitize the tumor cells to radiotherapy. Dichloroacetate, a PDK inhibitor that has the potential for such metabolic targeting, has been shown to reverse the Warburg effect by shifting glucose metabolism from glycolysis to mitochondrial oxidation and to inhibit tumor cell growth (9, 10). By combining with radiotherapy, dichloroacetate has been demonstrated to enhance the radiosensitivity of several tumor types in vitro (11–13). Interestingly, a previous study using 2 human cancer cell lines (colon adenocarcinoma and glioblastoma) demonstrated that dichloroacetate sensitized the efficacy of radiotherapy in vitro but attenuated radiotherapy-induced tumor growth delay in vivo (colon adenocarcinoma model; ref. 13). This paradoxical effect of dichloroacetate drove us to further investigate the efficacy and the mechanism of action of radiotherapy–dichloroacetate combination in both in vitro and in vivo glioblastoma models. Therefore, in the present study, we first examined the hypothesis that radiotherapy promotes glycolytic metabolism and then tested whether a reversal of the glycolytic phenotype will resensitize glioblastoma cells to radiotherapy using dichloroacetate. The findings of this study may have important implications for clinical trials aimed at preventing postradiotherapy metabolic changes and increasing the therapeutic index of radiotherapy for patients with glioblastoma.

Materials and Methods

Cell culture and chemical

Glioblastoma cell lines (U87, U251, LN229, DBTRG) were purchased from ATCC. U87 and U251 were cultured in MEM (Gibco) with 10% FBS and 2 mmol/L l-glutamine. LN229 was cultured in DMEM (Gibco) with 10% FBS and 2 mmol/L l-glutamine. DBTRG was cultured in RPMI-1640 (Gibco) supplemented with 10% FBS, 2 mmol/L l-glutamine, 25 mmol/L HEPES, and 1 mmol/L sodium pyruvate. Cell lines were obtained within the past 5 years and authentication was not performed. A patient-derived glioblastoma cell line RN1 (unmethylated, rs16906252 wild-type, and p53 mutant) was kindly provided by our collaborative researchers at the Queensland Institute of Medical Research (Brisbane, Queensland, Australia) and was cultured in advanced DMEM/F12 (Gibco) mixed with Neurobasal-A medium (Gibco; 1:1) supplemented with B-27 (1 ×), FGF (20 ng/mL), and EGF (20 ng/mL). Sodium dichloroacetate was purchased from Sigma.

Cell-cycle analysis

Cells were seeded in 6-well plates, followed by treatments as indicated. After treatments, cells were harvested and fixed in cold 70% v/v ethanol for at least 2 hours. Fixed cells were washed with PBS and stained in the dark with a solution containing propidium iodide (10 μg/mL), Triton X-100 (0.1%), and RNase (100 μg/mL) for 20 minutes at room temperature. DNA content was analyzed using a BD FACSCanto II flow cytometer and data analysis was performed using FlowJo (TreeStar Inc).

Mitochondrial superoxide production assays

Cells were seeded in 6-well plates, followed by treatments as indicated. MitoSOX Red (Invitrogen) was used to measure the level of mitochondrial superoxide production according to the manufacturer’s instructions. After staining, cells were trypsinized, centrifuged, and resuspended in HBSS (1 mL). Sytox Blue (Invitrogen; 1 μmol/L) was added to counterstain for nonviable cells. MitoSOX Red fluorescence was analyzed using BD FACSCanto II flow cytometer.

Extracellular flux assay

The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of glioblastoma cells were determined using the XF Extracellular Flux Analyzer (Seahorse Bioscience). XF 24-well plates were coated with Matrigel (BD biosciences) to allow cells to attach for this assay. Briefly, each well of the 24-well plate was coated with 50 μL diluted Matrigel (1:50 in PBS) overnight. The next day, cells were plated at a density of 30,000 cells (U87) and 50,000 cells (RN1) per well and allowed to attach overnight in culture media. The following day, the adherent cells were treated as indicated. On the assay day, cells were washed and fresh assay media were added. The cartridge was loaded to dispense 3 metabolic inhibitors sequentially as specific time points: oligomycin (inhibitor of ATP synthase, 1 μmol/L), followed by FCCP (a protonophore and uncoupler of mitochondrial oxidative phosphorylation, 1 μmol/L), followed by the combination of rotenone (mitochondrial complex I inhibitor, 1 μmol/L) and antimycin (inhibitor of complex III, 1 μmol/L). Specifically, the addition of oligomycin is used to measure the rate of proton leak across the inner mitochondrial membrane. The injection of FCCP is used to measure the rate of uncoupled respiration and is determined from the maximum respiration rate. The addition of rotenone–antimycin combination is to inhibit the transfer of electrons from iron–sulfur centers in complex I to ubiquinone and the oxidation of ubiquinol in the electron transport system (ETS), thereby inhibiting the oxidative phosphorylation. Basal OCR and ECAR were measured, as well as the changes in OCR caused by the addition of the metabolic inhibitors described above. Several parameters were deducted from the changes in OCR and mitochondrial reserve capacity was calculated by subtracting basal respiration from maximal respiratory capacity as described previously (14). A mirror plate was set up and treated identically in parallel with the assay plate. At the end of the assay, cells from the mirror plate were harvested, and the number of viable cells determined by flow cytometry was used to normalize the measurements.

Western blotting

Cells were seeded in 6-well plates, followed by treatments as indicated. Cells were lysed with RIPA buffer (Life Technologies), sonicated and centrifuged (14,000 rpm, 10 minutes, 4 °C). Protein concentration was measured using BCA Assay Kit (Pierce). Proteins (50 μg/sample) were separated via reducing 10% SDS-PAGE, and standard Western blotting procedures (15) were used to detect proteins of interest with the following primary antibodies: γ-H2AX (Cell Signaling Technology, CST #9718), HIF1α (CST, #3716), PDK-1 (CST, #3820), and β-actin (Abcam, ab8227). Images and densitometry were acquired by using ImageQuant TL software.

Glucose metabolism PCR array

Cells were plated in 6-well plates, followed by treatments as indicated. Total RNA from cells was extracted using RNAeasy Mini Kit (Qiagen). About 0.8 μg of total RNA was used for generating
cDNA with RT² First Strand Kits (Qiagen) and was analyzed by a glucose metabolism PCR array according to the manufacturer's instructions using Viia7 Real-Time PCR System (Life Technologies). All data were normalized to expression of untreated control in the corresponding sample.

Colony survival assay

Cells were seeded into 6-well plates and were allowed 24 hours for attachment followed by treatments as indicated. Culture medium was changed after 24-hour treatment. Plates were incubated for 2 weeks undisturbed. Colonies were gently washed with PBS followed by staining and fixation with crystal violet solution (0.5% in H₂O:methanol, 1:1) for 15 minutes. Stained colonies consisting of >50 cells were counted and the number was recorded. Plating efficiency was calculated as the number of colonies counted divided by number of cells seeded and normalized to the average plating efficiency of untreated samples. The average of these values was reported as "surviving fraction." For the combination therapy of dichloroacetate and radiotherapy, the surviving fraction was normalized to dichloroacetate treatment alone.

Mouse xenograft orthotopic model

The procedures for developing an orthotopic brain tumor model were performed according to a protocol approved by the Animal Care and Ethics Committee of University of New South Wales (Sydney, New South Wales, Australia). Briefly, female athymic nude mice (Balb/c, 8 weeks) were intracranially injected with 5 × 10⁴ U87 cells stereotactically in the right caudate putamen using the coordinates: 1 mm anterior, 1.5 mm lateral, and 3.0 mm below from the bregma.

Dichloroacetate treatment and radiotherapy

The U87-bearing mice were randomly divided into 4 groups of 8 to 10: vehicle-treated control, radiation alone, dichloroacetate alone, and combination treatment. Dichloroacetate was administered by oral gavage at 150 mg/kg/d from day 3 after tumor inoculation to the end of experiment. Mice in vehicle-treated group were administered 1% PBS followed by staining and fixation with crystal violet solution (0.5% in H₂O:methanol, 1:1) for 15 minutes. Stained colonies consisting of >50 cells were counted and the number was recorded. Plating efficiency was calculated as the number of colonies counted divided by number of cells seeded and normalized to the average plating efficiency of untreated samples. The average of these values was reported as "surviving fraction." For the combination therapy of dichloroacetate and radiotherapy, the surviving fraction was normalized to dichloroacetate treatment alone.

Statistical analyses

All analyses were performed using GraphPad Prism. Each independent experiment was performed with at least triplicate samples per treatment group. Results are expressed as mean ± SD of replicate values from 3 independent experiments or representative of 3 independent experiments presented as means ± SD of triplicate measurements. Statistical analysis was performed by 2-way ANOVA corrected by the Dunnett or Student t tests. Kaplan–Meier survival was compared using log-rank (Mantel-Cox) test. All tests of statistical significance were 2-sided and P < 0.05 was considered statistically significant.

Results

Radiotherapy shifts the glucose metabolism from oxidative phosphorylation to glycolysis and the addition of dichloroacetate blocks radiotherapy-induced glycolytic phenotype in glioblastoma cells

We first examined the expression of a panel of key genes involved in the regulation and enzymatic pathways of glucose and glycogen metabolism. Compared with untreated control, we observed that most of the glycolytic genes were upregulated in U87 cells irradiated with 6 Gy at 4 hours after radiotherapy. In particular, all the isozymes of PDK (PDK 1-4) were upregulated from 1.26- to 3.38-fold (Fig. 1A and Table 1). To determine whether HIF1α might be involved in this radiotherapy-induced glycolysis, Western blotting was performed to examine the protein level of HIF1α and its target PDK1. Compared with untreated control, treatment of U87 cells with 6 Gy radiotherapy significantly increased HIF1α and PDK1 expression up to 2.47- and 1.42-fold, respectively (Fig. 1B). To further confirm the radiotherapy-induced glycolysis, 2 metabolic parameters, OCR and ECAR, were measured using an extracellular flux assay. The bioenergetic profiles were obtained 4 hours postradiotherapy with increasing radiotherapy doses. As shown in Fig. 1C, the ECAR values of U87 cells after radiotherapy increased dose dependently, whereas a dose-dependent decrease in OCR was observed. By combining 10 mmol/L dichloroacetate with radiotherapy, the radiotherapy-induced glycolytic rate of U87 cells was attenuated significantly (P < 0.01) compared with cells treated with radiotherapy alone (Fig. 1D). Similar results were also obtained for the primary glioblastoma cell line RN1 (Fig. 1E).

Dichloroacetate treatment induces G₂–M cell-cycle arrest in glioblastoma cells

We next further examined whether the proliferation arrest from dichloroacetate treatment was associated with induction of cell-cycle arrest. Cells were treated with 25 and 50 mmol/L dichloroacetate for 24 hours, and cell-cycle profiles were analyzed using flow cytometry. Dichloroacetate treatment induced changes in the cell-cycle profiles of all tested glioblastoma cells (Fig. 2A and B). Specifically, after 24 hours of treatment with 25 mmol/L...
dichloroacetate, there was a slight increase (not significant) in the cells in G2–M phase in U87 and U251 cells and a 1.2-fold increase (P < 0.001) in RN1 cells (Fig. 2B). Significant increase in the mean percentage of all 3 tested cell lines in G2–M phase was observed when dichloroacetate dose was increased to 50 mmol/L (Fig. 2B). When compared with untreated control (U251, 8.8%; U87, 15.2%; RN1, 14.1%), dichloroacetate treatment increased the proportion of cells at G2–M phase to 35.5%, 34.7%, and 45.5%, respectively. A corresponding decrease in cells in G1 and S phases in all glioblastoma cell lines was also observed (Fig. 2B).

Dichloroacetate treatment depletes mitochondrial reserve capacity in glioblastoma cells

Because ionizing radiation exerts its cytotoxic effects predominantly through the generation of free radicals and subsequent oxidative stress, we investigated whether the mitochondrial

Table 1. The fold change of glucose/glycogen metabolism-related genes in irradiated U87 cells in comparison with untreated control

<p>| | | | | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ACLY</td>
<td>ACO1</td>
<td>ACO2</td>
<td>AGL</td>
<td>ALDOA</td>
<td>ALDOB</td>
<td>ALDOC</td>
<td>BPGM</td>
<td>CS</td>
<td>DLAT</td>
<td>DLD</td>
<td>DLST</td>
</tr>
<tr>
<td>2.17</td>
<td>1.09</td>
<td>2.69</td>
<td>1.12</td>
<td>1.98</td>
<td>1.34</td>
<td>2.62</td>
<td>1.48</td>
<td>1.27</td>
<td>1.06</td>
<td>1.15</td>
<td>2.67</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>ENO1</td>
<td>ENO2</td>
<td>ENO3</td>
<td>FBP1</td>
<td>FBP2</td>
<td>FH</td>
<td>G6PC</td>
<td>G6PC3</td>
<td>G6PD</td>
<td>HK2</td>
<td>HK3</td>
<td>IDH1</td>
</tr>
<tr>
<td>1.94</td>
<td>2.58</td>
<td>1.52</td>
<td>3.05</td>
<td>2.87</td>
<td>1.51</td>
<td>5.38</td>
<td>–1.35</td>
<td>2.69</td>
<td>1.7</td>
<td>1.46</td>
<td>1.56</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>GPI</td>
<td>GSK3A</td>
<td>GSK3B</td>
<td>GSY1</td>
<td>GYS2</td>
<td>H6PD</td>
<td>HK2</td>
<td>HK3</td>
<td>IDH1</td>
<td>IDH2</td>
<td>IDH3A</td>
<td>IDH3B</td>
</tr>
<tr>
<td>1.29</td>
<td>3.4</td>
<td>1.09</td>
<td>1.28</td>
<td>–1.07</td>
<td>4.29</td>
<td>1.78</td>
<td>–1.82</td>
<td>1.49</td>
<td>2.16</td>
<td>1.59</td>
<td>1.96</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>IDH3G</td>
<td>MDH1</td>
<td>MDH1B</td>
<td>MDH2</td>
<td>OGDH</td>
<td>PC</td>
<td>PCK1</td>
<td>PCK2</td>
<td>PDHA1</td>
<td>PDHB</td>
<td>PDK1</td>
<td>PDK2</td>
</tr>
<tr>
<td>1.92</td>
<td>1.04</td>
<td>1.54</td>
<td>1.52</td>
<td>2.17</td>
<td>1.56</td>
<td>–7.76</td>
<td>2.63</td>
<td>1.75</td>
<td>1.38</td>
<td>1.34</td>
<td>3.38</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>PDK3</td>
<td>PDK4</td>
<td>PDP2</td>
<td>PDP2</td>
<td>PFKL</td>
<td>PGAM2</td>
<td>PKG1</td>
<td>PKG2</td>
<td>PGLS</td>
<td>PGMI</td>
<td>PMG2</td>
<td>PMG3</td>
</tr>
<tr>
<td>1.31</td>
<td>1.26</td>
<td>1.49</td>
<td>1.39</td>
<td>3.48</td>
<td>5.52</td>
<td>1.76</td>
<td>–2.37</td>
<td>2.01</td>
<td>1.6</td>
<td>1.52</td>
<td>1.79</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>PHKA1</td>
<td>PHKB</td>
<td>PHKG1</td>
<td>PHKG2</td>
<td>PKLR</td>
<td>PRPS1</td>
<td>PRPS1L1</td>
<td>PRPS2</td>
<td>PYGL</td>
<td>PYGM</td>
<td>RBKS</td>
<td>RPE</td>
</tr>
<tr>
<td>1.99</td>
<td>1.85</td>
<td>2.75</td>
<td>–1.31</td>
<td>–1.12</td>
<td>2.14</td>
<td>–1.8</td>
<td>1.5</td>
<td>1.58</td>
<td>1.58</td>
<td>1.42</td>
<td>1.55</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>RPIA</td>
<td>SDHA</td>
<td>SHDB</td>
<td>SDHC</td>
<td>SDHD</td>
<td>SUCCL2</td>
<td>SUCCL1</td>
<td>SUCCL2</td>
<td>TALDO1</td>
<td>TKT</td>
<td>TPII</td>
<td>UGP2</td>
</tr>
<tr>
<td>1.65</td>
<td>1.77</td>
<td>1.64</td>
<td>–1.04</td>
<td>–2.18</td>
<td>1.61</td>
<td>1.33</td>
<td>1.78</td>
<td>1.39</td>
<td>1.76</td>
<td>1.6</td>
<td>1.51</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: This table shows the corresponding results of the heatmap (Fig. 1A). Glucose metabolism—Glycolysis: ALDOA, ALDOB, ALDOC, BPGM, CS, DLAT, DLD, DLST, FH, IDH1, IDH2, IDH3A, IDH3B, IDH3G, MDH1, MDH1B, MDH2, OGDH, PC, PCK1, PCK2, PDP1, PDP2, PDP3, TCA cycle: ACLY, ACO1, ACO2, CS, DLAT, DLD, DLST, FH, IDH1, IDH2, IDH3A, IDH3B, IDH3G, MDH1, MDH1B, MDH2, OGDH, PC, PCK1, PCK2, PDHA1, PDHB, SDHA, SDHB, SDHC, SDHD, SUCCL2, SUCCL1, SUCCL2, Pentose Phosphate Pathway: G6PD, H6PD, PGLS, PRPS1, PRPS1L1, PRPS2, RBKS, RPE, RPIA, TALDO1, TKT. Glycogen metabolism - Synthesis: GBE1, GYS3, GYS2, UGP2; Degradation: AGL, PMG1, PGMI, PGM3, PYGL, PYGM; Regulation: GSK3A, GSK3B, PHKA1, PHKB, PHKG1, PHKG2.
reserve capacity of glioblastoma cells would be altered by dichloroacetate treatment (Fig. 3A). After 24-hour treatment with 10 mmol/L dichloroacetate, the basal OCR of dichloroacetate-treated cells increased from about 4,200 pmole/min/10⁶ cells (untreated control) to about 5,400 pmole/min/10⁶ cells (Fig. 3B). By sequentially injecting oligomycin, FCCP, the mixture of antimycin and rotenone, complete mitochondrial profile was further revealed in U87 and RN1 cells (Fig. 3B and C). As dichloroacetate treatment did not obviously affect the maximal respiratory capacity of tumor cells, the mitochondrial reserve capacity of dichloroacetate-treated cells significantly decreased (P < 0.05; Fig. 3D). By increasing dichloroacetate concentration to 20 mmol/L, the reserve respiratory capacity was further significantly reduced to about 1,960 and 490 pmole/min/10⁶ cells in U87 and RN1 cells, respectively (P < 0.001; Fig. 3D).

**Radiotherapy in combination with dichloroacetate induces ROS production in glioblastoma cell**

As dichloroacetate increased oxidative stress by directing pyruvate into mitochondria, increasing electron transport chain activity, and thus generating more ROS, we proposed that this increased ROS could potentially further increase the ROS induced by radiotherapy. To test this hypothesis, glioblastoma cells (U251, U87 and RN1) were treated with dichloroacetate, radiotherapy alone, and the combination of 2 therapies followed by mitochondrial ROS level detection. For U251, 24-hour dichloroacetate treatment (25 and 50 mmol/L) led to a dose-dependent increase in mitochondrial ROS levels by 1.5- to 2-fold compared with untreated control (Fig. 4A). On the other hand, the mitochondrial ROS level of U251 cells treated with radiotherapy increased 1.3- (12 Gy) and 1.5-fold (18 Gy) compared with that of control cells (Fig. 4A). These values further increased when the dichloroacetate treatment was combined with radiotherapy. Specifically, the combination of dichloroacetate (25 and 50 mmol/L) and radiotherapy (12 and 18 Gy) dose dependently boosted the mitochondrial ROS level of U251 up to about 3.5-fold as opposed to control cells (Fig. 4A). Compared with U251, U87 cells are more resistant to dichloroacetate treatment and radiotherapy. Significant elevation of the MitoSox Red fluorescence was only induced with high-dose dichloroacetate treatment (50 mmol/L) and radiotherapy (18 Gy) alone (P < 0.01; Fig. 4B). A further increase in mitochondrial ROS production was observed when combined treatment was administered (Fig. 4B). Similar results were obtained from RN1 cells (Fig. 4C). These data suggest a higher capacity of the combination to induce mitochondrial ROS generation in comparison with individual treatments alone.

Figure 2.
Dichloroacetate (DCA) induces cell-cycle arrest in glioblastoma cells. A, representative flow cytometric histograms showing cell-cycle distribution in untreated control and 24-hour dichloroacetate-treated RN1 cells. B, cell-cycle analysis showing the percentage of cells in G1-, S-, and G2–M phases in untreated and dichloroacetate-treated glioblastoma cells (U87, U251, and RN1). Results are presented as means ± SD of 3 experiments performed in triplicates.

A, untreated control; DCA treated.

B, cell-cycle distributions (% of cells)
Dichloroacetate augments the DNA double-strand breaks induced by radiotherapy

As we observed that the combination of radiotherapy and dichloroacetate induced higher mitochondrial levels of ROS than each treatment alone, a further experiment was conducted to investigate the change in γ-H2AX levels, a hallmark of DNA damage, after treatment of radiotherapy and dichloroacetate. Western blotting for γ-H2AX production was measured 30 minutes after radiotherapy (4 Gy), 24-hour dichloroacetate treatment (50 mM), and the combination (pretreatment with dichloroacetate for 24 hours followed by radiotherapy). For U251, both radiotherapy and dichloroacetate treatment increased γ-H2AX production (4.75- and 2.71-fold) compared with untreated control, whereas the combination further induced γ-H2AX production up to 8.93-fold (Fig. 4D). Compared with U251, U87 is more resistant to the radiotherapy and dichloroacetate treatment. Either radiotherapy or dichloroacetate treatment as monotherapy showed similar levels of DNA damage with 1.29- and 1.82-fold γ-H2AX production. When the 2 treatments were combined, significantly higher γ-H2AX production (2.41-fold) was observed (Fig. 4D). Similar results were also obtained from RN1 with 2.08-, 1.52-, and 3.48-fold increase for radiotherapy, dichloroacetate, and the combination, respectively (Fig. 4D).

Dichloroacetate treatment synergizes with radiotherapy to impair the clonogenicity of glioblastoma cells

Clonogenic survival assays were conducted to examine whether dichloroacetate is a radiosensitizing agent for glioblastoma cells. U251, LN229, and DBTRG were irradiated with 2, 4, and 6 Gy after 6 hours of pretreatment with dichloroacetate (U87 and RN1 were not selected for this assay as they were not able to form clear colonies to be counted). As a single agent, dichloroacetate treatment induced a dose-dependent clonogenic inhibition in these glioblastoma cell lines (Supplementary Table S1). The
Clonogenicity of these cells was further impeded when treated with combination compared with dichloroacetate and radiotherapy alone (Fig. 5A–C). For U251 and DBTRG cells, dichloroacetate significantly synergized with all of the 3 tested radiotherapy doses to reduce the colony formation (Fig. 5A and C), whereas for LN229 cells, we observed an additive effect when dichloroacetate was combined with 2 and 4 Gy radiotherapy and synergy with 6 Gy (Fig. 5B).

The combination therapy extends the median survival of glioblastoma orthotopic model

We determined the efficacy of radiotherapy–dichloroacetate combination using a xenograft orthotopic mouse model bearing U87 cells. The dosage of radiotherapy (20 Gy in 10 fractions) and dichloroacetate (150 mg/kg/d) were selected on the basis of our previous studies showing either of them significantly delayed the growth of orthotopic U87 xenograft (data not shown). No toxicity was noticed in any of the treatment arms. Compared with vehicle-treated control (median survival time; MS, 29 days), dichloroacetate alone (MS, 31 days) prolonged the median survival of orthotopic model by 2 days ($P < 0.01$), whereas a more significant prolongation of median survival ($P < 0.001$) was found in radiotherapy alone (MS, 38 days) arm (Fig. 5D). Moreover, an even longer survival benefit was observed in radiotherapy–dichloroacetate combination arm (MS, 43 days) compared with each treatment alone ($P < 0.001$ vs. radiotherapy arm; Fig. 5D). The histologic analysis of the vehicle and treated tumors at day 23 confirmed tumor formation (Fig. 5E) and indicated significant reductions in the proliferation of the tumor cells, as reflected in number of cells staining for the proliferation marker Ki-67 (Fig. 5F and G).

**Discussion**

In the current study, several distinct lines of evidence indicate that the efficacy of radiotherapy was significantly enhanced by dichloroacetate treatment in glioblastoma cells. Our findings first confirmed an initial finding from others that a shift of glucose metabolism from oxidative phosphorylation to glycolysis occurred in glioblastoma cells following radiotherapy, despite...
Figure 5.
Efficacy of radiotherapy (RT) in combination with dichloroacetate (DCA) in vitro and in vivo. Clonogenic survival assay showing the combination of dichloroacetate and radiotherapy impairs the clonogenicity of U251 (A), LN229 (B), and DBTRG (C) cells. The surviving fraction curves of radiotherapy/dichloroacetate combination have been normalized to the curves of dichloroacetate-only. Any combination curve overlapping with the radiotherapy-only curve indicates additive effect, whereas any combination curve under the radiotherapy-only curve suggests synergistic effect. Results are representative of 3 experiments and presented as means ± SD of triplicate measurements. D, Kaplan-Meier survival analysis of xenograft orthotopic U87 model treated with dichloroacetate (150 mg/kg/d), radiotherapy (20 Gy in 10 fractions), and radiotherapy–dichloroacetate combination. E, representative images from H&E staining of mouse brain containing U87 tumor. b, normal brain parenchyma; t, tumor. F, representative images (40×) from Ki-67 staining of U87 orthotopic tumor. G, quantification of Ki-67-positive cells in U87 orthotopic tumors treated with 150 mg/kg/d dichloroacetate, 20 Gy radiotherapy, and the combination treatment. Results are expressed as the means ± SD, with n = 6 in each group. ns, not significant; **, P < 0.01; ***, P < 0.001. #, P < 0.001 versus 20 Gy radiotherapy; ns, not significant.
ample oxygen availability, which strongly support a switch to aerobic glycolysis. By combining dichloroacetate with radiotherapy, this glycolytic phenotype was reversed. We demonstrated that dichloroacetate alone moderately induced proliferation arrest at the G2–M phase and reduced the mitochondrial reserve capacity, indicative of sensitizing glioblastoma cells to the effect of radiotherapy. In addition, dichloroacetate and radiotherapy worked synergistically to induce elevated mitochondrial ROS level as well as γ-H2AX production, further confirming the mechanism of action of this combination. Finally, the clonogenicity of glioblastoma cells was impaired by the combination of dichloroacetate and radiotherapy, and the survival of an orthotopic mouse model was extended significantly by the combination therapy compared with each single treatment arm. All these findings above provide the evidence that modulating the glucose metabolism may serve as an effective approach to sensitize these malignant cells to radiotherapy.

Radiotherapy kills cancer cells primarily through free radical–induced DNA damage (16). However, it has been shown that this cornerstone of treatment acts as a double-edged sword. Radiotherapy has been linked with HIF1 activation/stabilization, which in turn activates transcription of numerous genes involved in angiogenesis, proliferation, pH regulation, and glycolytic metabolism (17–19). In particular, HIF1 not only initiates transcription of genes that encode transporters and enzymes regulating glucose metabolism but also activates the PI3K/AKT pathway that is involved in the regulation of metabolic shift to aerobic glycolysis. The underlying mechanism by which radiotherapy activates HIF1 was mostly investigated using in vitro preclinical model. It has been thought that HIF1 expression increases after radiotherapy due to reoxygenation and ROS elevation that induce HIF1α stabilization (20). We confirmed here using cultured glioblastoma cells that HIF1α was activated postradiotherapy as well as a panel of glycolytic genes. Interestingly, the upregulation of PDK1, a crucial mitochondrial enzyme that plays an important role linking glycolysis to oxidative phosphorylation and a direct target of HIF1α (21), was also observed. To further confirm this finding, we measured the glucose metabolism using an extracellular flux analyzer. A dose-dependent increase in the acid production was observed and a decline in the oxygen consumption occurred simultaneously.

It has been demonstrated that the glycolytic metabolism in malignancies highly correlates with radioresistance (22). Tumor cells predominantly using glycolysis counter the direct and indirect action of radiotherapy, that is radiotherapy–induced free radical and oxidative stress, by upregulating the endogenous antioxidant capacity through accumulation of pyruvate, lactate, and the redox couples glutathione/glutathione disulfide and NAD(P)H/NAD(P)+ (23). These macromolecules are also the products of glycolytic pathway which constitute an intracellular redox buffer network effectively scavenging free radicals and ROS, thus blunting the efficacy of radiotherapy. In this way, it is a potential strategy to overcome radioresistance by modulating tumor glucose metabolism and the cellular redox status. Glioblastoma is a deadly brain tumor for which there are limited therapies and chemoradiotherapy remains a serious problem to be conquered. Altered glucose metabolism in glioblastoma has been extensively investigated in vitro (5, 24, 25) and, more recently, in vivo in patients (26) and in human orthotopic glioblastoma models (27). These studies established that glucose is oxidized in the citric acid cycle in addition to confirming that there is a significant fraction of glucose that is shunted to lactate generation. Most recently, the same group of investigators published data in the orthotopic models and in patients demonstrating that acetate is also being oxidized in the citric acid cycle, which further confirms the significant role of mitochondria in glucose metabolism of glioblastoma cells. After exposing to ionizing radiation, the uncoupling of oxidative phosphorylation occurred (28), which in turn elevates the glycolytic rate, providing cancer cells with numerous advantages via the Warburg effect (29). In this respect, reversal of the Warburg effect offers dual therapeutic advantages by not only enhancing the effect of ionizing radiation through induction of oxidative stress but also lowering acid production resulted from the uncoupling of oxidative phosphorylation.

Dichloroacetate is a small-molecule PDK inhibitor that can penetrate blood–brain barrier and reverse the Warburg effect in cancer cells (30). It has been demonstrated with moderate antitumor activity (31–33) and to induce apoptosis in tumors of patients with glioblastoma by restoring the mitochondrial activity (34). Several studies have also used it as a radiosensitizer in lung (11), colorectal (13), and prostate cancer cells (12) in vitro. In the present study, we employed dichloroacetate as a radiosensitizer in both in vitro and in vivo glioblastoma models. Dichloroacetate was selected for combination with radiotherapy, as we proposed that by reversing the glycolytic phenotype with dichloroacetate and directing more pyruvate into mitochondrial oxidation (to produce more ROS), tumor cells would be more sensitive to radiotherapy. We found that concurrent use of dichloroacetate with radiotherapy indeed prevented the increase in glycolytic rate observed following radiotherapy alone. Our previous study demonstrated that dichloroacetate effectively induced a dose-dependent proliferation arrest of in vitro cultures of glioblastoma cells but had no significant effect on noncancerous cells (15). This result is in line with most of the studies that investigated the efficacy of dichloroacetate demonstrating that a therapeutic window exists although suprapharmacologic level of dichloroacetate (5–50 mmol/L) is necessary to halt the growth of cancer cells in vitro (9, 32, 33, 35). In the current study, we further analyzed the cell-cycle distribution after dichloroacetate treatment showing that dichloroacetate induced G2–M arrest and decreased the cell proportion in S-phase. These findings are consistent with some (35, 36), but not all studies (12, 37), indicating not all cancer types share the same mechanism of action after dichloroacetate treatment. As cancer cells are most sensitive to radiotherapy in the G2–M phase of the cell cycle whereas most resistance in the S-phase (38–40), our results suggest that dichloroacetate treatment can sensitize glioblastoma cells to radiotherapy by altering cell cycle distribution. Moreover, the mitochondrial reserve capacity, a measure of the ability of cells to resist oxidative stress (41), was reduced dose dependently after dichloroacetate treatment, which further confirms the role of dichloroacetate as a potential radiosensitizer to glioblastoma cells, even if the inhibition of glycolysis may also partly contribute to its role of radiosensitization in this setting. As ROS are synthesized during mitochondrial respiration, an increased rate of mitochondrial oxidation might exacerbate the ROS-induced DNA damage caused by radiotherapy (42, 43). It has been reported that dichloroacetate treatment induced ROS production both in vitro and in vivo (44). Recent evidence also
indicated that dichloroacetate increases autophagy in association with increased ROS production in colorectal cancer cells (45). Given ROS plays an important role in their individual mode of action of dichloroacetate and radiotherapy, we thus combined dichloroacetate with radiotherapy and demonstrated that the combination treatment not only increased the ROS production but also caused higher level of double-strand breaks than each treatment alone. These findings were further verified by our in vitro efficacy study demonstrating that the clonogenicity of glioblastoma cells was impaired synergistically when dichloroacetate was combined with radiotherapy. Ultimately, an orthotopic glioblastoma mouse model was used to test this hypothesis in vivo. A survival advantage was observed in the combination treatment arm compared with either single treatment arm and the results were confirmed with histologic examination showing a low percentage of Ki-67–positive cells in the combination treated tumors. These data again support our conclusion that dichloroacetate treatment may overcome the radioresistance of glioblastoma cells by reversing the glycolytic phenotype while synergizing with radiotherapy by stimulating the production of ROS. Notably, the discordance in the radiosensitizing effect of dichloroacetate was observed when it was tested in vitro and in vivo. In vitro, the effective radiosensitizing dose of dichloroacetate ranges from 25 to 50 mmol/L evidenced from the result of colony surviving assay as well as our mechanistic studies. In contrast, the efficacy of radiotherapy was sensitized by dichloroacetate with 150 mg/kg/d in vivo that gives much lower serum level of dichloroacetate. This finding is consistent with previous reports describing that this anti-metabolic compound has better in vivo efficacy than in vitro activity (37, 46, 47). The reasons for the limited antitumor effect of dichloroacetate with clinically relevant doses in vitro may lie in the complex cellular physiology and the immense excess of metabolites existing in cell culture media (46). Because of the fact that dichloroacetate has better in vivo activity than in vitro activity, it also has been suggested that there might be unique metabolic features and special growth pattern for solid tumors that are difficult to recapitulate in vitro and may be critical in determining the efficacy of this class of drugs (46).

Dichloroacetate has been used as an orphan drug for various acquired and congenital disorders of mitochondrial metabolism for decades and has recently been proved to be feasible and well-tolerated in patients with recurrent malignant gliomas in a recent phase I clinical trial (48). In addition, a recent study has tested the efficacy of dichloroacetate in a small cohort of patients with glioblastoma, suggesting metabolic modulation through PDK inhibition as a novel therapeutic strategy for the treatment of this devastating brain tumor (34). This study also observed that apoptosis was further increased in the glioblastoma stem cells treated with dichloroacetate plus temozolomide, indicating dichloroacetate may potentiate the effect of standard chemotherapy. Moreover, the use of dichloroacetate reverses the postradiotherapy glycolytic changes such that the efficacy of radiotherapy is enhanced without adding extra toxicity. Taken together, our findings warrant further evaluation of the combination of dichloroacetate and radiotherapy/temozolomide in clinical trials for newly diagnosed patients with glioblastoma.

References
18. Semenza GL. De...