Comparison of Spheroids Formed by Rat Glioma Stem Cells and Neural Stem Cells Reveals Differences in Glucose Metabolism and Promising Therapeutic Applications*

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Background: Cancer stem cells (CSC) are responsible for tumor resistance and relapse.

Results: Spheroids formed by CSC and neural stem cells (NSC) were compared by proteomics and functional studies. DCA, a glycolytic modulator, sensitizes CSC but not NSC to apoptosis.

Conclusion: DCA can eradicate glioma stem cells.

Significance: DCA could be a new efficient anti-CSC treatment in glioma.

Cancer stem cells (CSCs) are thought to be partially responsible for cancer resistance to current therapies and tumor recurrence. Dichloroacetate (DCA), a compound capable of shifting metabolism from glycolysis to glucose oxidation, via an inhibition of pyruvate dehydrogenase kinase was used. We show that DCA is able to shift the pyruvate metabolism in rat glioma CSCs but has no effect in rat neural stem cells. DCA forces CSCs into oxidative phosphorylation but does not trigger the production of reactive oxygen species and consecutive anti-cancer apoptosis. However, DCA, associated with etoposide or irradiation, induced a Bax-dependent apoptosis in glioma CSCs. The overexpression of BH3-only proteins (Bad, Noxa, and Puma), which in turn facilitates Bax-dependent apoptosis. Our results demonstrate that a small drug available for clinical studies potentiates the induction of apoptosis in glioma CSCs.

Mammalian cells generate ATP mainly through mitochondrial oxidative phosphorylation. However, cancer cells adopt an alternative metabolic pathway and increase their glycolytic activity through a process called the Warburg effect (1). The Warburg effect is necessary for cancer cells to resist oxidative stress and to adapt to hypoxic conditions (2). Thus, glycolysis could play an essential role in tumorigenesis during both the immortalization and transformation steps (3). It is still unknown and controversial whether or not the emergence of glycolysis in cancer is either (i) an early or late event or (ii) a genetically inherited dysfunction (essentially affecting mitochondria) or induced by metabolic modifications (mainly due to the hypoxic tumor environment) (4–6). Nonetheless, this metabolic transformation confers a selective growth advantage and/or resistance to apoptosis (7, 8) and thus might represent an important therapeutic target to be exploited (9, 10). The anti-cancer effects of modulators of glycolysis have been assessed in many different models (2). Dichloroacetate (DCA) is a small pharmacological inhibitor of pyruvate dehydrogenase kinase that promotes a shift in metabolism from glycolysis to oxidative phosphorylation (11). Bonnet et al. (12) have shown that DCA efficiently kills some cancer cells most likely because these cells are unable to produce ATP through mitochondria. Recently, the same group has shown that DCA induced apoptosis in glioblastoma multiforme (GBM) cells and thus could be beneficial to patients (13). Because gliomas are one of the most deadly malignant tumors with virtually no successful treatment (14), this discovery opens new promising therapeutic avenues. Michelakis et al. (13) have shown that DCA was also efficient against gliomas including a small population of tumor cells, called cancer stem cells (CSCs), which are highly resistant to cell death inducers and thus could be responsible for tumor recurrence (15). This is important because the eradication of CSCs represents a major therapeutic goal (15, 16). DCA has been shown to induce apoptosis in GBM CSCs but not in normal cells through a mechanism that remains elusive (13). Apoptosis is a complex process, which is often deregulated in cancer (17). Metabolism, and in particular oxidative phosphorylation,

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2 The abbreviations used are: DCA, dichloroacetate; CSCs, cancer stem cells; LDH, lactate dehydrogenase; NSC, neural stem cell; ROS, reactive oxygen species; 2-DG, 2-deoxyglucose; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDA, Limiting dilution assay; Glc-6-PD, glucose-6-phosphate dehydrogenase; DIGE, differential gel electrophoresis.

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is essential for Bax and/or Bak activation (18, 19); the core pro-apoptotic proteins of the Bcl-2 family (20). In this report, we have studied the influence of DCA on the apoptotic pathway and in particular the Bax activation pathway. We used a rat model, which allowed the comparison of the effect of this compound on neurons enriched in CSCs and neural stem cells (NSCs).

**EXPERIMENTAL PROCEDURES**

**Materials**

Cell culture material was obtained from Invitrogen. Unless stated otherwise, all chemicals were purchased from Sigma. Antibodies used were the following: Bax (2772, Cell Signaling), pan-actin (MAb1501R, Chemicon), Bad (610391, BD), Bim (AB17002, Chemicon), Noxa (AP1316a, Tebu), Puma (AP1317a, Chemicon), Foxo3 (07–702, Millipore), Bax 6A7 (731731, BD), and p53 (Pab 122, 554167, BD Bioscience). ABT737 was purchased from Euromedex, France. Results shown are the mean values of at least three independent experiments (±S.D.) unless expressively mentioned. The images were quantified using ImageJ software. Statistical analyses were performed using the GraphPad software (Student unpaired t test, * p < 0.05; ** p < 0.01).

**Methods**

**Cell Culture**—Glioma primary cultures (P7 and M7) were obtained from Sprague-Dawley rats following antenatal ENU induction as described in Poulilquen et al. (21). The enrichment of CSC was obtained by culturing the cells in defined medium (DMEM/Ham’s F-12 containing 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, N2- and B27-supplement, 20 ng/ml of EGF, 25 ng/ml of bFGF, and 2 μg/ml of heparin).

NSCs were obtained from E14 Sprague-Dawley rat embryos. Briefly, embryo brains were rinsed, freed from meninges, incubated with trypsin 0.25% for 15 min at 37 °C then with 10 μg/ml of DNase I for 10 min at 37 °C. Cells obtained were plated in complete medium (DMEM (glucose 1 g/liter) containing 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin) overnight at 37 °C. The non-adherent cells recuperated and were cultured in defined medium (DMEM containing 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, N2- and B27-supplement, 20 ng/ml of EGF, 25 ng/ml of bFGF, and 2 μg/ml of heparin (supplemental Fig. S1)).

**RT and Quantitative PCR**—Cells were washed twice in PBS, then total RNA was isolated using the RNeasy MiniKit (Qiagen) following the manufacturer’s instructions and DNase I treatment. After RNA quantification by NanoDrop (NanoDrop ND-1000, Thermo Fisher Scientific, Waltham, MA), 1 μg of RNA was reverse transcribed using Reverse Transcriptase AffinityScript (Agilent-Stratagene, Massy, France) for cDNA synthesis. Quantitative real-time PCR assays were performed and monitored in triplicate using a MX4000 multiplex quantitative PCR system (Agilent-Stratagene). The PCR contained 40 ng of cDNA in a reaction volume of 25 μl (1× Brilliant II SYBR Green Quantitative PCR master mix, 200 nM reverse and forward primers, and 30 nM SYBR Green). Thermocycling conditions were 95 °C for 10 min followed by 40 cycles at 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min. Gene expression values were normalized to the housekeeping gene (Ubiquitin) and relative expression values were calculated based on the comparative ΔΔCt method. The following primers were used:

| Gene     | Sense Primer                  | Antisense Primer                  |
|----------|-------------------------------|-----------------------------------|
| Foxo3    | 5’-CGCAAAAACGAAGAACTCAGA-3’   | 5’-AACTTCACCTCTCCACACGAT-3’       |
| Foxo3    | 5’-GGGGATTGGTGGTAAACTCAGA-3’  | 5’-AACTTCACCTCTCCACACGAT-3’       |
| p53      | 5’-TCTGGACGACAGCCGCTACG-3’    | 5’-AACTTCACCTCTCCACACGAT-3’       |
| Bax      | 5’-CAGGATGGGTTGCTGACT-3’      | 5’-AACTTCACCTCTCCACACGAT-3’       |
| Bcl-2    | 5’-TCTGGACGACAGCCGCTACG-3’    | 5’-AACTTCACCTCTCCACACGAT-3’       |
| N2       | 5’-TCTGGACGACAGCCGCTACG-3’    | 5’-AACTTCACCTCTCCACACGAT-3’       |
| B27      | 5’-TCTGGACGACAGCCGCTACG-3’    | 5’-AACTTCACCTCTCCACACGAT-3’       |

Microarray Analysis

Gels images were processed using Progenesis SameSpots software. Gene expression values were normalized to the housekeeping gene (Ubiquitin) and relative expression values were calculated based on the comparative ΔΔCt method. The following primers were used:

| Gene     | Sense Primer                  | Antisense Primer                  |
|----------|-------------------------------|-----------------------------------|
| Foxo3    | 5’-CGCAAAAACGAAGAACTCAGA-3’   | 5’-AACTTCACCTCTCCACACGAT-3’       |
| Foxo3    | 5’-GGGGATTGGTGGTAAACTCAGA-3’  | 5’-AACTTCACCTCTCCACACGAT-3’       |
| p53      | 5’-TCTGGACGACAGCCGCTACG-3’    | 5’-AACTTCACCTCTCCACACGAT-3’       |
| Bax      | 5’-CAGGATGGGTTGCTGACT-3’      | 5’-AACTTCACCTCTCCACACGAT-3’       |
| Bcl-2    | 5’-TCTGGACGACAGCCGCTACG-3’    | 5’-AACTTCACCTCTCCACACGAT-3’       |
| N2       | 5’-TCTGGACGACAGCCGCTACG-3’    | 5’-AACTTCACCTCTCCACACGAT-3’       |
| B27      | 5’-TCTGGACGACAGCCGCTACG-3’    | 5’-AACTTCACCTCTCCACACGAT-3’       |

**Cell Proliferation Assays**—Cells were cultured in 96-well plates and 5000 cells per well were seeded. After 24 h, the cells were treated with DCA (0.075 g/liter, drinking water) and/or etoposide (200 μg/mouse, intraperitoneal injection, 5 of 7 days).

**Two-dimensional DIGE Experiments**—Cell pellets were resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS) and proteins were further precipitated using the Two-dimensional Clean-Up kit (Amersham Biosciences). Four biological replicates (50 μg) were used under each condition. Fluorescent CyDye labeling of proteins for DIGE was performed according to the manufacturer’s instructions (GE Healthcare). Isoelectrofocusing was performed on 18-cm pi 3–11 NL Immobilon Dry strip gels (GE Healthcare) and SDS-PAGE were run using the Ettan DALTsix system (GE Healthcare). CyDye-labeled gels were scanned in an Ettan DIGE Imager (GE Healthcare). Gels images were processed using Progenesis SameSpots software (Nonlinear Dynamics Ltd.) for spot detection and statistical analysis. Spots considered for further analyses were chosen according to the following criteria: p < 0.05 (one-way analysis of variance test), fold-change >1.3, q < 0.05 and power >80% (False Discovery Rate approach). Spots were recovered using the Ettan Spot picker (GE Healthcare) and individually treated with the ProteoExtract All-in-one Tryptsin Digestion kit (Calbiochem) according to the manufacturer’s instructions. Peptide digests were concentrated on C18-Zip Tips (Millipore) and eluted with 1.5 μl of α-cyano-4-hydroxycinnamic acid matrix solution (50% acetonitrile, 0.05% trifluoroacetic acid) onto a MALDI-TOF target plate (Opti-TOF 384 well Insert, Applied Biosystems). Peptide spectra acquisition was done on a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems). After screening the sample position in MS-positive reflector mode using 1500 laser shots, the fragmentation of automatically selected precursors was performed at collision energy of 1 kV.
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using air as the collision gas (pressure of 2 × 10⁻⁶ torr). MS spectra were acquired between m/z 800 and 4000. Up to 12 of the most intense ion signals having a S/N > 12 were selected as precursors for MS/MS acquisition. Peaklist generation and protein identification were performed with ProteinPilot™ Software version 2.0 (Applied Biosystems) using the Paragon algorithm. Each MS/MS spectrum was searched for all species against the Uniprot_Sprot_20080123 database. The searches were run using the fixed modification of iodoacetamide-labeled cysteine parameter enabled. Other parameters such as tryptic cleavage specificity, precursor ion mass accuracy, and fragment ion mass accuracy are MALDI 4800 built-in functions of ProteinPilot software. The ProteinPilot software calculates a confidence percentage (the unused score), which reflects the probability that the hit is a false positive, meaning that at 95% confidence level, there is a false positive identification chance of about 5%.

Protein Extraction and Immunoblotting—Total proteins were extracted in 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS supplemented with protease inhibitor mixture from Roche Diagnostics (Mannheim, Germany). Protein concentration was determined using the Bradford assay (Bio-Rad). Protein extracts were separated by SDS-PAGE, transferred onto PVDF membrane (Millipore), and revealed with ECL (Roche France). HRP-conjugated secondary antibodies were from Bio-Rad. PVDF membrane (Millipore), and revealed with ECL (Roche Diagnostics). Primary antibodies were used at 1:1000 dilution. HRP-conjugated secondary antibodies were from Bio-Rad. Quantification was performed with ImageJ software from at least three independent experiments.

Flow Cytometry—After treatment, the cells were dissociated, washed, and labeled. The level of 6A7 Bax was assessed by flow cytometry measuring the FL1 fluorescence in a FACScalibur (BD Biosciences). Analyses were performed using the CellQuest software (BD Biosciences). A primary gate based on physical parameters (forward and side light scatter) was set to exclude dead cells or debris. The background level was estimated by omitting the primary antibody.

LDH Activity Measurement—Cells were plated at a density of 10⁵ cells/100 µl in 96-well plates. The cells were lysed by the addition of 10 µl of lysis solution incubated at 37 °C for 45 min. 50 µl of supernatant was used to determine the LDH concentration according to the manufacturer’s instructions (Promega, France).

Glucose-6-phosphate Dehydrogenase (Glu-6-PD) Activity—Cells were harvested and washed with PBS, then sonicated for 2 min in 100 µl of PBS and centrifuged for 15 min at 13,000 × g. Protein concentration was assessed using Bradford assay (Bio-Rad). Glc-6-PD activity was assessed in samples by spectrophotometric measurement of NADP⁺ reduction using a Roche/Hitachi 917 chemistry analyzer (substrate: 100 mM Tris, pH 7.8, 4 mM Mg²⁺, 1 mM NADP⁺)

MTT Assay—Cells were plated in 96-well plates at a density of 1.5 × 10⁴ cells per well and cultured in the presence of 2-deoxyglucose (25 or 35 mM), 3-bromopyruvate (20 or 40 mM), or antimycin A (1 or 3 µM) for 24 h. 10 µl of 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added per well and the plate was incubated at 37 °C for 3 h. 100 µl of dimethyl sulfoxide was added and the plate was read at 570 nm. The linearity between the number of viable cells and MTT assay in the experimental conditions used was verified by direct counting of cells after eosin staining.

ROS Measurement—Reactive Oxygen Species (ROS) were measured as previously described (22).

Respiratory Parameters in Intact Cells—Respiratory parameters were investigated in intact NSCs and P7 cells by polarography in 2-ml glass chambers using a two-channel, high-resolution Oxygraph respirometer (Oroboros, Innsbruck, Austria) as described previously (23). The routine respiration rate of intact cells (3–5 × 10⁶ cells at 37 °C in 2000 µl of DMEM) was determined by measuring the linear rate of oxygen consumption in intact cells. Then, oligomycin (8 µg/ml) was added, and the resting respiration, i.e., the nonphosphorylating respiration rate, was recorded. The maximal uncoupled respiration rate was then obtained using 0.5–10 µM uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone. Finally, respiration was inhibited by addition of 3 µM antimycin.

Mitochondrial Enzymatic Activity Measurements—The activity of citrate synthase and cytochrome c oxidase was determined as described previously (24).

Caspase Assay—DEVDase activity was measured as previously described (25).

Immunostaining—For immunostaining, neurospheres were dissociated and 50 µl of cell suspension were adhered to Super-frost slides. Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% SDS for 10 min, blocked with 3% BSA for 20 min, and then incubated with antibodies against Foxo3 or p53 for 1 h followed by secondary Alexa antibodies (Invitrogen) for 1 h. Cells were mounted in Prolong Gold plus DAPI (Invitrogen) to stain nuclei. The labeling was acquired using a Zeiss microscope adapted with an Apotome camera (Zeiss Axiovert 200M inverted microscope and AxioVision 4.6 program). Colocalization of Foxo3 or p53 with DAPI was measured in an average of 10 cells per field using Metamorph software (version 7.6.5.0). The acquisition of imaging data were done at the Cellular and Tissular Imaging Core Facility of Nantes University (MicroPiCell).

RESULTS

Characterization of Rat Neural and Cancer Stem Cell Spheres—Under our culture conditions (see “Experimental Procedures”), some isolated cancer cells grew as floating spheroids morphologically comparable with the neurospheres formed by rat NSCs, although with a less ordered structure (Fig. 1A). The rat glioma cancer cells were able to form serial neurospheres and limiting dilution assays (LDA) (26, 27) indicated that the percentage of stem cells in the different cancer cell populations ranged from 2.9 to 3.9 (4.6% for M7, 3.9% in P7, both glioma primary cultures, and 2.9% in the C6 cell line). A percentage that was comparable although slightly inferior to that found in NSCs (i.e. 9.1%) (Fig. 1B).

We also verified that glioma-derived spheroids expressed stem cell markers (nanog, oct-1, nestin, sox-2, Ms1, and the self-renewal marker Bmi1) (Fig. 1, C and D) often overexpressed in human CSCs (28). In addition, injection of P7 neurospheres drastically increased the cancer cells tumorigenicity compared with that of adherent cells (Fig. 1E). Of note, the same observation was made with M7 neurospheres (data not
Thus, the spheroids obtained from ENU-induced rat gliomas exhibited characteristics of CSCs. A two-dimensional DIGE and MS/MS proteomic comparison between NSCs and glioma spheroids (see "Experimental Procedures") allowed the identification of 132 differential spots among which, 44 distinct proteins were characterized. Using the Ingenuity Pathways Knowledge Base, significant networks, functions, and canonical pathways associated emerged from these differentially expressed proteins. As shown in Fig. 2A and Table 1, glycolysis and pyruvate metabolism were significantly different between CSCs and NSCs. Lactate dehydrogenase and glucose-6-phosphate dehydrogenase activities confirmed that glioma CSCs essentially metabolized glucose through a nonoxidative pathway (Fig. 2B). We also measured the expression of the glucose transporter Glut-1 and found that it was up-regulated in glioma CSCs as compared with NSCs (Fig. 2C). This up-regulation was consistent with the fact that rat glioma CSCs were completely dependent on glucose for their growth in vitro (Fig. 2D), whereas NSCs were capable to use alternative substrates, even if their proliferation rate was lower than that of CSCs. Finally, as expected, glucose replacement by galactose, supplemented or not with pyruvate, could not support CSCs growth in vitro (Fig. 2D). To further confirm the glycolytic CSCs metabolism, we inhibited the mitochondrial oxidative pathway using antimycin A, and as expected, this specifically inhibited NSCs growth under normoxia although having no effect on CSCs proliferation (supplemental Fig. S2).

Effect on DCA on Glioma and Neural Neurospheres—Recent results have suggested that DCA shifted the metabolism of pyruvate from glycolysis toward oxidation in the mitochondria through pyruvate dehydrogenase kinase inhibition and have powerful anti-cancer activities (12, 13). This has been particularly illustrated in the case of human gliomas. Spheres were treated with DCA (1 mM), which provoked an increase in the maximal uncoupled respiration rate (i.e. compared with O2 consumption in the presence of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone in CSCs but not in NSCs (Fig. 3A). Of note, this increase could be attributed neither to an increase in the amount of mitochondria (as illustrated by the citrate synthase activity measurement) nor to an increase in the mitochondrial complexes activity (as illustrated by the COX IV activity measurement) (supplemental Fig. S3). DCA has been reported to induce a sharp increase in ROS in human gliomas (12). We did not observe any modification in ROS production in rat CSCs (Fig. 3B); nevertheless, under these conditions, DCA decreased the activity of LDH in CSCs but not in NSCs (Fig. 3C), indicating nonetheless a metabolic shift in CSCs. We...
conclude that the neurospheres obtained from the rat glioma provided an excellent model to analyze and compare the effect of DCA in rat NSCs and glioma CSCs.

Next, we tested the effect of DCA on the ability of rat NSCs and CSCs to generate neurospheres. LDA and experimental estimation of the stem-like cell number in the population (28) showed that DCA gradually reduced the CSC ability to form spheroids, whereas that of NSCs were unaffected (Fig. 3D and supplemental Fig. S4). However, this phenomenon could not be uniquely attributed to the inhibition of glycolysis as a treatment with 2-DG affected both CSC and NSC neurosphere formation (Fig. 3D and supplemental Fig. S4).

DCA Decreased the Resistance to Apoptosis in CSCs Treated with Etoposide or Irradiation—CSCs are known to be resistant to etoposide- or irradiation-induced apoptosis (29). To assess the effect of DCA on apoptosis, neural and glioma spheroids were treated with increasing concentrations of DCA over 48 h and cell viability was monitored as described under "Experimental Procedures." As shown in Fig. 4A, little or no effect of DCA on cell viability and proliferation was observed under these conditions even at the highest concentration (i.e. 1 mM), including when a prolonged treatment induced a decrease in CSC sphere proliferation (supplemental Fig. S5). Next, NSC- and CSC-enriched spheres were treated with etoposide (50 μM...
for 12 h) or irradiated (5 gray); these conditions alone did not affect their survival (Fig. 4B). However, a DCA pretreatment drastically increased the number of dead cells in cancer spheroids (Fig. 4B). Caspase activity (i.e. DEVDase) was measured under these different conditions and the combined treatments DCA/etoposide or DCA/irradiation were more apoptogenic than the single regimen (Fig. 4C). Of note, similar results were obtained with CSCs obtained from human glioblastoma multiforme (supplemental Fig. S6).

DCA Induced a Bax-dependent Apoptosis—To verify the implication of Bax in the above observed cell death, the expression of Bax was targeted using small hairpin RNA (supplemental Fig. S7). C6CSC and P7CSC were treated with two shRNAs targeting Bax (shBax1 and shBax2) and a control scramble shRNA. Both shBax efficiently inhibited the expression of Bax (supplemental Fig. S7). These cells were treated as previously described with DCA and etoposide or irradiation. As illustrated in Fig. 5, A and B, the knockdown of Bax expression abolished both cell death and caspase activation.

To understand how DCA could alter Bax-dependent apoptosis in CSCs, the expression of some BH3-only proteins (Bad, Bim-EL, Noxa, and Puma), shown to be induced by glycolytic inhibitors, was measured. The data in Fig. 5C show that the expression of Bad, Noxa, and Puma were increased in DCA-treated CSCs compared with untreated cells, whereas that of Bim was unaffected. Proteins of the Bcl-2 family are essential partners of apoptosis. ABT-737 is a drug, which has been demonstrated to efficiently inhibit the anti-apoptotic members of this family, such as Bcl-2 or Bcl-XL (30), and has demonstrated promising effects in different cancer cells (30). As shown in Fig. 5D ABT-737 alone had a small effect on CSCs and NSCs survival but its combination with DCA augmented cell death in CSCs but not in NSCs. This result suggests that proteins of the Bcl-2 family are involved in DCA-enhanced apoptosis in CSCs. Taken together, our results suggest that DCA was able to increase the expression of some BH3-only proteins in CSCs, but not sufficiently to activate Bax. This effect appears to be specific because 2-DG did not increase the expression of Bad, Puma, and Noxa in these cells (supplemental Fig. S8).

DCA Up-regulates p53 and Foxo3 Expression—Foxo3 and p53 are known to be transcription factors for Noxa, Puma, Bim, and Bad (31–33). To assess if these proteins were implicated in the DCA pathway, we analyzed by quantitative PCR their expression. As shown in Fig. 6A, an increase in the expression of these two transcription factors was observed upon treatment with DCA in CSCs but not in NSCs. For p53, the expression increased 24- (C6CSC) and 10-fold (P7CSC), whereas that of Foxo3 increased 4- (C6CSC) and 31-fold (P7CSC). This up-regu-

FIGURE 3. Effect of DCA on NSC and CSC metabolism. A, oxygraph analyses of NSCs and P7CSC treated or not with DCA (1 mM, 48 h). Respiratory parameters were investigated in intact cells as described previously (23). The respiration rate was normalized to the quantity of protein in each experiment. Data are the mean ± S.D. of 3 independent experiments (**, p < 0.01). B, NSC, C6CSC, and P7CSC were cultured in defined medium with or without DCA (1 mM, 48 h) and the production of ROS was assessed using a fluorescent dye (H2DCF-DA), as previously described (22). C, cells were treated by DCA (1 mM, 48 h), then LDH activity was measured as described under “Experimental Procedures.” D, DCA (1 mM) or 2-DG (25 mM) was added to P7CSC and NSCs and LDA experiments were done on days 4, 6, 8, 10, and 12 for P7CSC and on days 6 and 12 for NSCs. The kinetics of the content of stem cells in the population is shown on the graph. FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone.
lation was confirmed by Western blot analyses (Fig. 6B). The cellular localization of these transcription factors is essential for their pro-apoptotic functions. As shown in Fig. 6C, Foxo3 localization, assessed by immunostaining, was modified in DCA-treated P7CSC compared with untreated cells because Foxo3 translocated into the nucleus. On the other hand, no modification in Foxo3 localization was observed in DCA-treated NSCs (44% ± 3.8 versus 42% ± 9.5). The percentage of nuclear Foxo3 upon DCA treatment was increased from 33 ± 3 to 53 ± 5%. Fig. 6C showed a similar modification in p53 localization in DCA-treated CSCs where the percentage of p53 in the nucleus increased from 27 ± 4 to 42 ± 2%. These results suggest that DCA may increase the transcriptional activity of p53 and Foxo3, thereby facilitating their pro-apoptotic activities.

To investigate the role of apoptosis and p53 in the cell death process, we used Pifithrin-α, a chemical inhibitor of p53 (34) and analyzed its effect on DCA/etoposide treatment. As expected, Pifithrin-α inhibited DCA/etoposide-induced apoptosis but also affected the formation of neurospheres in LDA (supplemental Fig. S9, A and B). Similarly, a knockdown in the expression of Foxo3 by siRNA rendered CSCs resistant to DCA-induced apoptosis (supplemental Fig. S9C).

Effect of DCA on Tumor Growth in Vivo—To study the effect of DCA in vivo, $5 \times 10^4$ P7CSC cells were injected into the flanks of nude mice and the animals were treated or not with DCA (diluted in the drinking water) and/or intra-peritoneal etoposide injections. Tumor size was measured twice a week for 1 month. As shown in Fig. 7, P7CSC tumor growth was rapid in mice treated or not with etoposide (681 mm$^3$ ± 70.6 versus 653 mm$^3$ ± 79.1 in 40 days) whereas, tumor growth decreased in mice treated with DCA (322 mm$^3$ ± 54.8) and even more when the mice were treated with DCA plus etoposide (67 mm$^3$ ± 11.2). Similar results were obtained when tumors were injected intracranially (data not shown).

DISCUSSION

Rat glioma models recapitulate several of the human GBM histopathological features and are reproducible and easily available (35). We have recently reported that the ENU-induced rat glioma model was an excellent model to monitor tumor growth and establish new therapeutic strategies (21, 36). Here, we show that this model allows a direct comparison between rat normal and cancer cells that could be relevant to the human pathology.

The CSC theory postulates that cells that harbor the primordial oncogenic mutations are rare, long-lived and slow-dividing and thus, evade most anti-cancer therapies. These cells are able to give rise to cells with distinct phenotypes if the right stimulus is provided. In gliomas, the most common adult brain tumors, CSCs have been involved in tumor resistance to treatment because these cells are highly radio- and chemoresistant (29). Due to the “stemness properties” of these cells, it has been postulated that CSCs could arise from pathological stem cells or dedifferentiated progenitors. This contention has been strengthened by the fact that current markers of glioma CSCs are similar to that of NSCs and because, like the normal counterpart, they are able to form neurospheres in vitro. In addition, tumor stem cells grown under sphere forming conditions are a
more reliable model for primary tumors than the classical conditions in the presence of serum (37).

In this work, we have performed a comparative study between NSC and CSC neurospheres obtained from normal rat brain and chemo-induced brain tumors, respectively (Fig. 1). We found that cells derived from these tumors or the established rat glioma cell line C6 exhibited different amounts of neurosphere-forming cells, which are otherwise highly tumorigenic cells (Fig. 1). Compared with cells grown in the presence of serum, the neurospheres formed by CSCs expressed several stem cell markers (i.e. Oct-1, nanog, Bmi-1, and nestin) in comparable amounts to that found in NSCs (Fig. 1). However, CSC neurospheres expressed less Sox2 and more Msi1 than the NSC neurospheres (Fig. 1). Consistent with the highly tumorigenic properties of CSCs, spheres formed highly proliferative tumors in animals compared with serum-cultured cells (Fig. 1).

Having established that spheres formed from rat brain tumors had CSC properties, we then compared these cells to NSCs using a two-dimensional DIGE and mass spectrometry-based strategy. We identified 20 proteins that had an altered expression in normal versus cancer neurospheres (Table 1). This analysis revealed that the main differences between NSCs and CSCs were associated with the glycolytic pathway and this was confirmed by both analytic and functional studies (Fig. 2). This phenomenon has been long known in rapidly dividing cancer cells as the Warburg effect (1). Of note, astrocytes have been shown to rely more on glycolysis than neurons, a feature that could facilitate their transition toward malignant stages (38).

The observation that the glycolytic pathway was overstimulated in CSCs compared with NSCs prompted us to analyze the response of these cells to glucose deprivation and/or to the inhibition of glycolysis. We show that DCA, a pharmacological modulator of glucose metabolism (12), induced apoptosis, in combination with DNA damaging agents, in CSCs but did not harm cognate normal stem cells. Interestingly, Michelakis et al. (13) have shown that CD133+/H11001 human glioblastoma multiforme cells (i.e. putative CSCs) were also sensitive to DCA but no molecular mechanism of action of this drug was reported.

We found that DCA could rapidly deplete the neurosphere-forming population and reduce tumor growth in vivo (Figs. 3 and 7). The effect of DCA was specific for CSCs, as the glycolytic metabolism and ability to form neurospheres of NSCs were unaffected by DCA, contrary to the results obtained with the classical inhibitor of glycolysis, 2-DG (Fig. 3). Bonnet et al. (12) have shown that DCA induced the production of ROS, however, this does not appear to be the case in CSCs (Fig. 3).

We further investigate the effect of DCA on CSCs and found that it induced a rapid and efficient cell death in CSCs only in the presence of an additional apoptotic stimulus (Fig. 4). This
cell death was Bax-dependent. In addition, DCA induced the expression of Bad, Noxa, and Puma, contrary to that observed with 2-DG (Fig. 5 and supplemental Fig. S7). Of note, ABT-737, an antagonist of Bcl-2 and Bcl-Xl but not of Mcl-1, enhanced DCA-induced apoptosis (Fig. 5). Also consistent with these results, DCA induced the expression of known transcription factors of these BH3-only proteins, namely Foxo3a and p53 (Fig. 6). Glycolysis affects the Bcl-2 family, especially the BH3-only proteins such as Bad and Puma (39–42). Recently it has been shown that 2-DG efficiently eliminated cancer cells in combination with ABT-737, through the Mcl-1/Bak axis (43, 44). This implies that 2-DG and DCA sensitizes toward apoptosis via different routes. The specific ability of DCA to deplete the neurosphere-forming population in rat glioma CSCs might be related to the induction of p53. It was shown that p53 inhibited the renewal of stem/progenitor cells in NSCs and glioma CSCs (45). The specific induction of p53 in CSCs might explain the divergent effect on the sphere-forming ability induced by DCA on NSCs and CSCs. Of note, p53 inhibition rescinded the sensitization of CSCs to apoptosis; p53 inhibition also delayed the CSCs depletion induced by DCA, however, long term p53 inhibition by itself induced a decrease in CSCs in the population (supplemental Fig. S9). The mechanism explaining this depletion will be the aim of further investigations.

Our results suggest that the dependence on glycolysis may occur early during tumorigenesis because it is already present in CSCs. Thus, the Warburg effect is likely to be a major event during tumorigenesis, not as a symptom but as one of its primary causes. Taken together, our results provide the rationale for the association of DCA and conventional treatments to eradicate CSCs and thus to prevent tumor relapse while preserving NSCs. Because, recent results have shown that DCA was active against several other types of cancer (see for example, Ref. 46), including undifferentiating neuroblastoma (47), it would be interesting to investigate whether CSCs are also a preferential target in tumors of different origins.

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