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Clinically relevant doses of chemotherapy agents reversibly block formation of glioblastoma neurospheres

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Abstract

Glioblastoma patients have a poor prognosis, even after surgery, radiotherapy, and chemotherapy with temozolomide or 1,3-bis(2-chloroethyl)-1-nitrosourea. We developed an *in vitro* recovery model using neurosphere cultures to analyze the efficacy of chemotherapy treatments, and tested whether glioblastoma neurosphere initiating cells are resistant. Concentrations of chemotherapy drugs that inhibit neurosphere formation are similar to clinically relevant doses. Some lines underwent a transient cell cycle arrest and a robust recovery of neurosphere formation. These results indicate that glioblastoma neurospheres can regrow after treatment with chemotherapy drugs. This neurosphere recovery assay will facilitate studies of chemo-resistant subpopulations and methods to enhance glioblastoma therapy.

Keywords

chemotherapy; cancer stem cells; DNA damage; temozolomide; BCNU; glioblastoma; neurosphere

1. Introduction

Temozolomide (TMZ) is the most common chemotherapy drug used to treat glioblastomas [1]. 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, carmustine) is an older drug that surgeons now deposit in the tumor bed as dissolvable wafers [2]. Both of these drugs alkylate DNA at multiple sites, including the O⁶ position of guanine, which can result in futile cycles of DNA repair and, ultimately, cell death. [3].

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Conflict of interest

None declared.

Even after aggressive treatment that eliminates most of the tumor load, these tumors recur in an average of 6.9 months after initial treatment [4]. Tumor regrowth implies that glioblastomas include a population of cells that are resistant to therapy and maintain the ability to proliferate. Recently, a population of cells with stem cell-like properties was identified in brain tumors [5], and several laboratories have reported that these cells are relatively resistant to chemotherapy and radiation [6;7].

Neurosphere cultures are heterogeneous, and only a fraction of cells are capable of sphere formation. These cells are classified as neurosphere initiating cells (NICs) [8;9;10]. To quantify the NICs, normal or transformed cells are plated at low density in defined medium and after 1–2 weeks, the numbers of neurospheres are scored [11;12]. For normal neural cells, this assay was developed to assess stem cells [8], but is now thought to also detect early progenitor cells [13;14;15]. It is not known whether there is a similar ambiguity for tumor NICs. Spheroid-based drug screens for other tumor types are under development [16].

The glioblastoma cells used for this assay are grown as neurospheres in defined medium to prevent differentiation. Unlike serum-supplemented cultures, glioblastoma neurosphere cells form invasive brain tumors in immunodeficient mice [17]. In addition, based on expression profiling, neurosphere cultures resemble glioblastoma tumors from patients more closely than serum-supplemented cultures [17].

Using the neurosphere-formation assay, we found that clinically relevant doses of BCNU or TMZ inhibit neurosphere formation. For four of five cell cultures, neurosphere formation resumes following a recovery period; dissociation of these initial spheres allows robust formation of secondary spheres. BCNU and TMZ induce S and/or G₂/M cell cycle arrest, which partially reverses by seven days post-treatment. Collectively, these results indicate that neurosphere formation is highly sensitive to chemotherapy drugs, and in some cases, the NICs may enter a reversible cell cycle arrest. This reversible cell cycle arrest may protect the NICs from chemotoxicity [18;19;20;21], allowing regrowth of some cultures after chemotherapy. In addition, an *ex vivo* TMZ-treated culture that resumes sphere formation is also capable of tumor initiation as subcutaneous xenografts. This model for the survival and recovery of cultured glioblastoma neurosphere cells may provide insights for tumor recurrence *in vivo*.

2. Materials and Methods

2.1 Cell culture

In a previous study, PTEN ^{-/-} neural precursor cells [22] were infected with a retrovirus bearing the human mutant receptor EGFRvIII, and we refer to these as EGFRvIII PTEN ^{-/-} cells [23]. These transformed cells formed glioblastoma-like tumors in immunodeficient mice, and we established the aggressive PET2 line from one of these tumors. As a control, PTEN ^{+/+} neural precursor cells were infected with an empty GFP MSCV-XZ066 virus [24]. We refer to these as GFP PTEN ^{+/+} cells. A major advantage of these mouse cultures is the easy comparison of normal and transformed cells.

The human adherent glioblastoma cell line U373MG (obtained from Dr. Larry Recht, Stanford University) was converted to a floating neurosphere culture, U373NS, by growing cells in 20% FBS DMEM/F12 until confluent and then in defined serum-free DMEM/F12 medium supplemented with B27 (GIBCO Carlsbad, CA), 20 ng/ml bFGF (Invitrogen Carlsbad, CA) and 20 ng/ml EGF (Invitrogen). These cells formed neurospheres after they were in serum-free media for approximately one month. U373NS neurospheres could be maintained for >30 passages by mechanical trituration. Primary human glioblastoma cultures (GS7-2 and GS9-6) were established from resected tumor tissue with Institutional

Review Board approval. The tumors were cut into small sections and dissociated with trypsin. Dissociated tumor cells were grown as neurosphere cultures in DMEM/F12 medium supplemented with B27, 20 ng/ml bFGF and 20 ng/ml EGF. The primary glioblastoma cultures were passaged using basic solution to dissociate the spheres [25]. The characterization of GS7-2 was reported by Dr. Cochran and co-workers [26]. All experiments using primary human GBM cultures were carried out with cells from passages 10–20.

All of the glioblastoma cultures, but not GFP PTEN +/+, formed subcutaneous tumors in immunodeficient mice (data not shown). As reported for other glioblastoma neurosphere cultures [27], fetal bovine serum induced astroglial differentiation of GFP PTEN +/+, PET2, U373NS, GS7-2, and GS9-6 (data not shown). All cultures used in this paper are O⁶-methylguanine DNA methyltransferase (MGMT) negative (data not shown).

As part of the basic characterization of these lines, we determined if the tumor suppressor p53 was mutated. The mRNA was extracted using a QuickPrep micro mRNA purification kit (GE Healthcare, Piscataway, NJ). One hundred ng of mRNA was incubated for 2 hrs at 37°C with 1 µl of Oligo(dT)_{12–18} (500 µg/ml), 4 µl of 5X First strand cDNA buffer, 2 µl of 0.1 mM DTT and 1 µl of 10 mM dNTP Mix (final volume 20 µl). Then 1 µl of SSII Reverse Transcriptase (200 U) was added and incubated for 50 min at 42°C, followed by heat inactivation for 15 min at 70°C. The p53 cDNA (2 µl) was amplified with the Platinum Taq DNA High Fidelity Polymerase (Invitrogen Carlsbad, CA). The primers for amplification of the human p53 cDNA were 5'-ATGGAGGAGCCGAGTCAGAT-3' and 5'-TGCGCCGGTCTCTCCAGGAC-3'; 5'-AAGGAAATTTGCGTGTGGAGT-3' and 5'-CAGTCGGAGTCAGGCCCTTCT-3'. The primers for amplifying the mouse p53 cDNA were 5'-TTGGGACCATCCTGGCTGTAG-3' and 5'-ATAAGGTACCACCACGCTGTG-3' for exon1–5, 5'-ATGGTGATGGCCTGGCTCCTC-3' and 5'-CAGTTCAGGGCAAAGGACTTC-3' for exon 6–8, 5'-AAGTCCTTTGCCCTGAAGTGC-3' and 5'-GACCGGGAGGATTGTGTCTCA-3' for exon 9. The PCR products were analyzed by automated DNA sequencing in both directions (GENEWIZ, South Plainfield, NJ).

PET2 showed only a C132W mutation, which is not active as judged by a WAF1 reporter construct (data not shown). GS9-6 had the wild type p53 sequence. The GS7-2 showed only a mutated F113V sequence. Amino acid 113 was mutated in four tumors (Sanger Cosmic Database), including one F113V mutation in a glioblastoma. We did not test U373NS cells because the parent line, U373MG, is already known to lack functional p53 [28].

2.2 Primary and secondary neurosphere formation

Neurospheres were mechanically triturated into single cell suspensions or disassociated with basic solution [25] and plated at 3,000 cell/ml of defined medium. To measure the effect of chemotoxicity, BCNU (Sigma-Aldrich St. Louis, MO) or TMZ (a gift from Dr. Michael Glantz) were added to these cultures at a range of concentrations. Neurospheres with at least 25 cells/sphere were counted after 7 or 10 days after addition of drug. Then, to measure recovery from drug treatment, cultures were supplemented with fresh defined media, and neurospheres were counted a second time, 14 or 20 days after drug treatment. The primary glioblastoma cultures (GS7-2 and GS9-6) formed spheres slightly slower than the other cultures. As a result, we allowed about 10 days for sphere formation by the primary human cultures and 7 days for the U373NS line and mouse cultures. For the secondary neurosphere assay, treated cells were collected after recovery on day 14 or 20, and dissociated into single cells. Cells from each well were replated into 6-well plates in 2 ml of defined medium. Secondary neurosphere formation was quantified by counting the number of neurospheres (>25 cells) after 7 or 10 days.

2.3 Proliferation assay

Single-cell suspensions were plated at 5,000 cells/well in a 96 well plate with or without BCNU or TMZ. After one week, viable cells were measured using colorimetric assays following the manufacturer's instructions (Promega Corp, Madison, WI). In initial experiments with the mouse cells, we used 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT). In later experiments with the human cell cultures, we used 3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) because of its greater solubility. In both cases, mitochondria in viable cells reduce these compounds to a formazan, which is detected by absorbance in a Microplate reader.

2.4 Subcutaneous xenografts with ex vivo TMZ treatment

U373NS neurospheres were dissociated and plated at 2.5×10^4 cells/ml in defined media and treated with DMSO or 200 μ M TMZ. On day 7, the cells were dissociated and 3×10^6 live cells were re-suspended in 100 μ l PBS. Cells were subcutaneously injected into the right flank of female athymic nude mice. The mice were observed for tumor formation for 100 days post-injection and were sacrificed when the tumor reached a volume of 1.5 cm³. These procedures were approved by the University of Massachusetts Medical School IACUC.

2.5 Comet assay

DNA damage in neurosphere cells with and without TMZ or BCNU treatment was measured by a comet assay, using protocols and reagents provided by Trevigen Inc. (Gaithersburg, MD). Briefly, single cells were embedded in agarose, lysed and treated with an alkaline solution to unwind and denature the DNA. The agarose-embedded cells were then subjected to electrophoresis. Cleaved DNA fragments caused by single or double stranded breaks migrate out of the nuclei and are detected using SYBR Green. For each experiment, at least 75 cells were examined with a Zeiss Axiovert S100 microscope, and DNA was quantified with OpenLab 4.0 software (Improvision Inc., Waltham, MA). The *comet tail moment* is calculated as the product of the tail length and the relative pixel intensity of the comet tail compared with the pixel intensity and the area of the nucleus. Comet tail moments are proportional to the extent of DNA damage in the cell [29]. Comet tail moments of treated cells were normalized to the comet tail moments of untreated cells.

2.6 Flow cytometry

Flow cytometry was used to measure the percentages of CD133+, CD15+ and A2B5+ cells following treatment with chemotherapy drugs. Cells were suspended in PBS containing 5% fetal calf serum, and stained with antibodies against CD133, CD15, and A2B5. Mouse cells were stained with monoclonal anti-mouse CD133 antibody (1:100; Clone 13A4, Chemicon/Millipore Corp (Billerica, MA), followed by a secondary PE-conjugated antibody (1:200; Jackson ImmunoResearch, West Grove, PA). For human cells, we used a PE-conjugated CD133 antibody (1:10 dilution; Clone AC141, Miltenyi Biotec, Auburn, CA). CD15 and A2B5 were detected with conjugated antibodies: CD15-APC (1:5; Clone HI98, Pharmingen/BD Biosciences, San Diego, CA) and A2B5-FITC (1:450; Clone A2B5-105(7), Millipore Corp., Billerica, MA). Marker expression was measured using a Becton Dickinson FACSCalibur (BD Biosciences, Franklin Lakes, NJ). Data was analyzed using FlowJo 7.2.2 software (Tree Star Inc., Ashland, OR).

To analyze cell cycle profiles, cells were fixed with 95% ethanol for at least 24 hrs and washed with apoptosis buffer (PBS, 1% Triton X-100 and 2 mM MgCl₂) to permeabilize the cells. The cells were then stained for 30 min prior to analysis with the FACSCalibur with 50

µg/ml propidium iodide (Sigma-Aldrich St. Louis, MO) in PBS, 50 µg/ml RNase and 2 mM MgCl₂. Data were analyzed using ModFit LT 3.0 software (Verity Software House, Topsham, ME).

3. Results

3.1 Neurosphere formation is inhibited at lower concentrations of chemotherapy drugs than those required to inhibit bulk cell proliferation

To determine how chemotherapy drugs affect neurosphere formation, we measured neurosphere formation in non-transformed and glioblastoma neurosphere cultures without or with treatment with BCNU or TMZ. Because of their rapid decay in aqueous solution [1;30], these drugs likely persist only a few hours, but their effects on the tumor cells last longer [31]. After 7 or 10 days, we counted the number of neurospheres that formed without or with drug treatment. For all of these cultures, BCNU and TMZ inhibited neurosphere formation in a dose-dependent manner (Fig. 1A–D).

To determine the sensitivity of bulk or total cells to these chemotherapy drugs, we performed MTT or MTS assays to measure the number of viable cells at the time that the neurospheres were counted. Inhibition of bulk cell proliferation, compared with neurosphere formation, required higher doses of chemotherapy drugs for all of these cell cultures (Fig. 1). To facilitate this comparison, we calculated the drug concentrations at which neurosphere formation was inhibited by 50% (IC₅₀), the drug concentrations at which bulk cell numbers were decreased by 50% (MTS/MTT IC₅₀) and the concentration at which bulk cells numbers were decreased by 90% (MTS/MTT IC₉₀) (Fig. 2). GFP PTEN +/+ and PET2 cells had similar sphere IC₅₀ values for treatment with BCNU or TMZ. The MTT IC₅₀ and MTT IC₉₀ values were both 5-fold (BCNU) and 1.4–2.0-fold (TMZ) higher for PET2 than for GFP PTEN +/+, indicating that normal GFP PTEN +/+ bulk cells are more sensitive to BCNU and TMZ than the transformed PET2 cells (Figure 2A–B). The sensitivities of the glioblastoma cultures to BCNU and TMZ varied, but there was one consistent finding. The concentration of chemotherapy drug required to inhibit neurosphere formation is much less than that required to inhibit bulk cell proliferation. The MTS IC₅₀'s for the inhibition of bulk cell proliferation following BCNU treatment were 2.5 to 40 fold higher than those for sphere formation. For TMZ, the MTS IC₅₀'s were 5 to 240 fold higher than sphere IC₅₀'s. Finally, the IC₉₀ values were substantially higher than the sphere IC₅₀'s for both BCNU and TMZ (Fig. 2), indicating that sphere formation is inhibited at much lower doses of drug than that required to induce substantial toxicity.

3.2 Recovery of neurosphere formation after treatment with chemotherapy drugs

To determine if neurosphere formation resumes after chemotherapy treatment, cultures were counted after a recovery period. Experiments were done with cultures treated with 50 µM BCNU or 200 µM TMZ, which greatly decreased initial neurosphere formation (Fig. 1). Specifically, initial neurospheres were counted 7 or 10 days (based on culture growth rate) after the addition of chemotherapy drugs. Then, fresh medium was added, and the culture was allowed to recover for an additional 7–10 days of in the absence of drugs. Neurospheres counted after the recovery period include both the initial spheres formed by day 7 or 10 and new spheres that formed during the recovery period (Fig. 3A).

The cell cultures varied greatly in their recovery from the two chemotherapy drugs. Normal GFP PTEN +/+ cells did not recover from BCNU treatment, but did recover from TMZ treatment (5-fold increase in sphere formation approaching statistical significance, $p < 0.13$, Fig. 3B), indicating that 200 µM TMZ is less toxic to normal neural precursor cells compared with 50 µM BCNU. The transformed PET2 cells showed dramatic 13- and 17-

fold increases in the number of spheres following BCNU and TMZ treatments, respectively (Fig. 3A). For GS7-2, there was a dramatic 5-fold increase in neurospheres during the recovery period after 50 μ M BCNU treatment (Fig. 3C). In contrast, the GS7-2 neurospheres did not increase significantly after treatment with 200 μ M TMZ. For both chemotherapy drugs, the number of neurospheres for GS9-6 cultures did not increase in the recovery period. For U373NS, the number of neurospheres increased nearly 2-fold during the recovery period for both BCNU and TMZ (Fig. 3C).

As a measure of NIC self-renewal, we also measured secondary sphere formation. The lack of recovery from 50 μ M BCNU for GFP PTEN $+/+$ cells was confirmed by the lack of secondary spheres. GFP PTEN $+/+$ cells formed secondary spheres following 200 μ M TMZ treatment, confirming that 200 μ M TMZ was less toxic to these normal neurosphere cells compared with 50 μ M BCNU (Fig. 3D). PET2 and U373NS cells formed secondary spheres following treatment with both drugs. GS7-2 formed secondary sphere formation following treatment with 50 μ M BCNU but not 200 μ M TMZ. GS9-6 did not form secondary neurospheres after treatment with 50 μ M BCNU or 200 μ M TMZ.

In summary, for some cell cultures, inhibition of neurosphere formation by the chemotherapy drugs is reversible. Following a recovery period, there is a robust increase in neurosphere formation, indicating that NICs were viable but did not proliferate immediately after chemotherapy treatment. Secondary sphere formation confirmed that some NICs avoided chemotoxicity and restored the neurosphere cultures.

To further study the effects of chemotherapy drugs on glioblastoma cells, we measured the percentages of cells expressing the stem cell markers CD133, CD15, and A2B5 by flow cytometry (Supplementary Table 1)[5; 32; 33; 34]. The baselines and changes in expression levels of the stem cell markers CD133, A2B5 and CD15 following chemotherapy treatment varied between neurosphere cultures. For untreated cultures, the percentage of CD133+ cells varied between 2 and 91%. Chemotherapy treatment either had no effect or slightly decreased the percentage of CD133+ cells. These results are consistent with other publications demonstrating that the current markers used for labeling glioblastoma stem cells are not sufficient and yield different results [34; 35; 36]. The neurosphere assay, on the other hand, provided a much more consistent finding. Based on decreased neurosphere formation, we conclude that neurosphere formation is more sensitive to chemotherapy drugs than bulk or total cell proliferation.

3.3 TMZ-treated cultures maintained their tumorigenicity

After demonstrating that NICs treated with clinically relevant TMZ concentrations are capable of repopulating the culture, we determined whether the cells that persist are still tumorigenic. U373NS cultures were treated with DMSO control or 200 μ M TMZ and injected subcutaneously into nude mice. The control DMSO treated cells formed palpable tumors in an average of 15 days for 7/7 xenografts. TMZ treatment increased the latency of tumor formation, however, the tumor incidence was similar to the DMSO control xenografts. Palpable tumors formed for 6/7 TMZ-treated U373NS xenografts in an average of 43 days (Fig. 4).

3.4 Normal and transformed neurosphere cultures accumulate DNA damage

We found that the transformed PET2 neurosphere cultures were more resistant to treatment with chemotherapy drugs than the normal GFP PTEN $+/+$ neurosphere cells (Fig. 1–3). To determine if this resistance was due to lack of DNA damage, we used the comet assay to measure accumulated DNA damage in untreated GFP PTEN $+/+$ and PET2 neurosphere cells and cells at 7 days after treatment with BCNU or TMZ. Following treatment with 50

μM BCNU, GFP PTEN $+/+$ and PET2 cells had 6.1 ± 0.4 and 4.7 ± 1.6 fold increases in comet tail moments, respectively, demonstrating a similar degree of DNA damage (Fig. 5A). Treatment with $200 \mu\text{M}$ BCNU resulted in even greater increases (34 ± 12 fold) in comet tail moment for PET2 cells. Following treatment with $200 \mu\text{M}$ TMZ, GFP PTEN $+/+$ and PET2 cells comet tail moments increased 2.0 ± 0.2 and 10 ± 4 fold, respectively (Fig. 5B). Contrary to the similar extent of DNA damage following $50 \mu\text{M}$ BCNU treatment, PET2 cells accumulated more DNA damage than GFP PTEN $+/+$ cells following treatment with $200 \mu\text{M}$ TMZ. PET2 cells treated with $800 \mu\text{M}$ TMZ also sustained more damage (29 ± 10 fold) than GFP PTEN $+/+$ cells (4 ± 2 fold). Therefore, we conclude that the relative resistance of the PET2 cells compared with GFP PTEN $+/+$ cells is not due to a lack of DNA damage.

3.5 Glioblastoma neurosphere cells undergo death or cell cycle arrest in response to chemotherapy drugs

To further characterize the recovery from chemotherapy drugs, we carried out propidium iodide flow cytometry on untreated and treated cultures (Fig. 6). In response to $50 \mu\text{M}$ BCNU or $200 \mu\text{M}$ TMZ, PET2 and U373NS cells showed accumulation of cells in S and/or G_2/M when analyzed three days post-treatment. We next tested whether cells resumed a normal cell cycle profile. At 7 days post-treatment, the profiles for PET2 cells resembled those for untreated cells except for increased debris. The profile for BCNU-treated U373NS cells 7 days post-treatment showed a smaller fraction of cells accumulated in G_2/M as well as increased debris. For TMZ-treated U373NS cells, the G_2/M accumulation had dissipated, but there was residual S phase accumulation. Overall, we see a smaller fraction of cells arrested in G_2/M at seven days compared with three days for PET2 and U373NS. These results indicate that BCNU and TMZ induce some cells to enter reversible cell cycle arrest. The recovery of neurosphere formation in the recovery week (Fig. 3), suggests that NICs are included in the reversibly arrested fractions.

4. DISCUSSION

To more effectively treat glioblastomas, we must target the tumor-initiating cells that are resistant to current therapies. To determine the effects of chemotherapy drugs on normal and glioblastoma neurosphere cells, we measured neurosphere formation and bulk cell proliferation after treatment with BCNU or TMZ. The MTT/MTS IC_{50} 's were 2.5–240-fold greater than the neurosphere IC_{50} 's, and the MTT/MTS IC_{90} values for which there was substantial cell death were 16–400-fold greater than the neurosphere IC_{50} 's. The relative sensitivity of neurosphere formation in response to chemotherapy was consistent for a non-transformed neurosphere culture and four glioblastoma cultures. Hence, we find that chemotherapy drugs efficiently target NICs. For U373NS cells, the MTS dose-response curves were not monotonic. We do not know the origin of this phenomenon, but dose-response curves for chemotherapy drugs are often non-monotonic [37].

To determine if NICs are capable of neurosphere formation after chemotherapy treatment, we devised an assay to measure the recovery of neurosphere formation. We treated neurosphere cultures with doses of chemotherapy drugs that drastically inhibit neurosphere formation, counted the neurospheres after 7 or 10 days, and again counted neurospheres after an additional 7 or 10 days. Surprisingly, although chemotherapy treatment resulted in drastic inhibition of initial sphere formation, some neurosphere cultures substantially increased the number of neurospheres during this recovery period. In addition, neurosphere cultures that recovered after treatment, were dissociated, replated and found to form secondary spheres. Glioblastoma neurosphere cells previously treated with chemotherapy also maintained tumorigenicity. Collectively, these results demonstrate that some NICs treated with clinically relevant doses of chemotherapy avoided chemotoxicity and were capable of restoring the culture.

The expression of stem cell markers used in previous studies to isolate NICs varied greatly among our GBM cultures. In addition, there was no consistent increase or decrease in marker expression following chemotherapy treatment. Finally, even though the percentage of GS9-6 cells expressing CD133 and CD15 is much higher than that for U373NS, the U373NS culture, but not GS9-6, recovers from chemotherapy treatment. The percentage of CD133+ cells for GS7-2 is 18-fold greater than that for U373NS, but does not show enhanced recovery compared to U373NS. In agreement, several recent studies have shown that CD133 is not a reliable marker for cancer stem cells [38;39;40;41]. In summary, stem cell marker expression varies among tumor cultures, does not consistently change following chemotherapy treatment and does not predict which glioblastoma cultures survive treatment with chemotherapy drugs. Neurosphere formation appears to be more accurate than marker expression for evaluation of NIC survival after chemotherapy treatment.

Two GBM neurosphere cultures that resumed neurosphere formation after chemotherapy treatment also underwent reversible cell cycle arrest as assayed by propidium iodide flow cytometry. Flow cytometry indicated that BCNU and TMZ induced a substantial accumulation of cells in G2/M or S phase three days after treatment. By seven days post treatment, some cells died and appeared as debris, but other cells resumed cycling and repopulated the culture. Cell cycle arrest in response to a toxic environment may preserve and maintain viable cell populations that are capable of resuming proliferation [7;18;20;21].

An intriguing question is why in some cases, recovery occurs for one of the drugs and not the other. For example, the GS7-2 neurospheres show a robust recovery from 50 μ M BCNU but not from 200 μ M TMZ. This may be a result of the substantially different responses to BCNU-induced and TMZ-induced DNA damage [42;43].

The GFP PTEN $+/+$ and PET2 cells allow us to make a direct comparison of a normal and transformed pair of cultures. The transformed PET2 cells are more resistant to these chemotherapy drugs. The comet assays showed that following treatment with 200 μ M TMZ, the PET2 cells had more DNA breaks than the GFP PTEN $+/+$ cells. These results suggest that the PET2 cells do sustain DNA damage but are more resistant to the effects of DNA damage than the GFP PTEN $+/+$ cells. It has previously been noted that expression of the EGFRvIII receptor or loss of PTEN makes cells resistant to apoptosis [44;45;46].

A critical question is how these experiments relate to drug treatments used in the clinic. In patients, BCNU is usually administered in a wafer inserted by the surgeon into the tumor bed. The concentrations of BCNU within several centimeters of the wafer are quite high (1–7 mM) and toxic [2;47]. More distal from the wafers, lower doses of BCNU would be less toxic and may induce reversible cell cycle arrest, enhancing tumor recurrence. In contrast, the *in vivo* serum concentration of TMZ is about 15 μ M, and the concentration in the brain is estimated to be about 5 μ M [48;49;50], which would inhibit proliferation of tumor-initiating cells but not induce tumor cell death. Indeed, 200 μ M TMZ, which is never reached *in vivo*, was not sufficient to kill all of the NICs. The BCNU levels in the vicinity of the wafer are more than sufficient to kill both NICs and bulk cells. The TMZ levels are only sufficient to inhibit NIC proliferation and, thereby, may slow tumor growth. Hence, BCNU close to the wafer may work by a quite different mechanism compared with BCNU distal from the wafer or TMZ.

The neurosphere recovery observed in this study is clinically relevant. Our finding that the NICs can resume proliferating after treatment with clinically relevant doses of chemotherapy drugs may be similar to the recurrence that occurs in glioblastoma patients. Our experiments provide an *in vitro* model to analyze the NICs that are capable of recovery after chemotherapy treatment. Because the neurosphere cultures resemble patients' tumors [17],

this model will facilitate experiments testing new drugs to target the chemoresistant population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
bFGF	basic fibroblast growth factor
EGF	epidermal growth factor
MGMT	O ⁶ -methylguanine DNA methyltransferase
MMT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
MTS	3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NIC	neurosphere initiating cells
PTEN	phosphatase and tensin homologue deleted on chromosome 10
TMZ	temozolomide

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Fig. 1.

Neurosphere formation is inhibited at a much lower concentration of chemotherapy drug than that required to inhibit bulk cell proliferation. BCNU and TMZ were added to dissociated neurosphere cultures. After 7 or 10 days, we counted the number of neurospheres and calculated the percentage of cells that formed new neurospheres (A–D). We also performed MTT or MTS assays to measure bulk cell proliferation (E–H). Dose-response curves were determined for mouse normal cells (GFP PTEN +/+) and glioblastoma PET2 cells (A–B and E–F). Similar results were obtained with human glioblastoma lines (C–D) and (G–H).

Fig. 2.

Chemotherapy drugs more efficiently inhibit neurosphere formation than bulk cell proliferation. We calculated the concentrations of chemotherapy drug required to inhibit neurosphere formation by 50% (sphere IC₅₀), the concentrations of drug required to inhibit bulk cell proliferation by 50% (MTS/MTT IC₅₀) and the concentrations required to inhibit bulk cell proliferation by 90% (MTS IC₉₀)(A–D). Also, normal neurosphere bulk cells are more sensitive to chemotherapy drugs than the transformed PET2 cells (A–B).

Fig. 3.

Inhibition of neurosphere formation by chemotherapy drugs can be reversible. (A) Neurospheres were counted 7 or 10 days after chemotherapy treatment (blue line), and then after an additional 7 or 10 days (recovery period, red line) to allow neurosphere-initiating cells to proliferate in the absence of drug (B–C). The initial measurements from day 7 or 10 are labeled with the cell culture name. Measurements after the recovery period on day 14 or 20 are labeled with the cell culture name and “R”. To analyze self-renewal, these neurospheres were dissociated and replated. Secondary spheres were counted after an additional 7 or 10 days (green line)(D). Although chemotherapy treatment greatly reduced

initial sphere formation, some cultures were capable of recovery and secondary sphere formation. *, $p < 0.05$.

Fig. 4.

Glioblastoma cells treated in culture with TMZ can form tumors. U373NS cells were treated in culture with a DMSO negative control or 200 μ M TMZ. After 7 days, cells (3×10^6) were injected subcutaneously into immunodeficient mice. The TMZ-treated cells formed tumors in immunodeficient mice with increased latency compared to control-treated cells.

Fig. 5.

The comet assay was used to assess DNA breaks in GFP PTEN +/+ and PET2 cells. Data are presented as the ratio of the comet tail moment following treatment to the comet tail moment with no treatment. (A) Following treatment with 50 μM BCNU, GFP PTEN +/+ and PET2 cells accumulated a similar number of DNA breaks. PET2 cells showed more DNA breaks after treatment with 200 μM BCNU. (B) PET2 cells accumulated more DNA breaks compared with GFP PTEN +/+ following treatment with 200 or 800 μM TMZ.

Fig. 6. PET2 and U373NS glioblastoma cell cultures show transient cell cycle arrest in response to chemotherapy drugs. Cell cycle profiles were determined 3 and 7 days after addition of drug. PET2 and U373NS cells show S and/or G₂/M phase accumulation 3 days post-treatment. This accumulation partially reverses by 7 days post-treatment, and in some cases, debris (sub G₁) appears.