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Research Article

The regrowth kinetic of the surviving population is independent of acute and chronic responses to temozolomide in glioblastoma cell lines

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ABSTRACT

Chemotherapy acts on cancer cells by producing multiple effects on a cell population including cell cycle arrest, necrosis, apoptosis and senescence. However, often a subpopulation of cells survives and the behavior of this subpopulation, which is responsible for cancer recurrence, remains obscure. Here we investigated the in vitro short- and long-term responses of six glioblastoma cell lines to clinically relevant doses of temozolomide for 5 days followed by 23 days of recovery, mimicking the standard schedule used in glioblastoma patient for this drug. These cells presented different profiles of sensitivity to temozolomide with varying levels of cell cycle arrest, autophagy and senescence, followed by a regrowth of the surviving cells. The initial reduction in cell number and the subsequent regrowth was analyzed with four new parameters applied to Cumulative Population Doubling (CPD) curves that describe the overall sensitivity of the population and the characteristic of the regrowth: the relative end point CPD (RendCPD); the relative Area Under Curve (rAUC); the Relative Time to Cross a Threshold (RTCT); and the Relative Proliferation Rate (RPR). Surprisingly, the kinetics of regrowth were not predicted by the mechanisms activated after treatment nor by the acute or overall sensitivity. With this study we added new parameters that describe key responses of glioblastoma cell populations to temozolomide treatment. These parameters can also be applied to other cell types and treatments and will help to understand the behavior of the surviving cancer cells after treatment and shed light on studies of cancer resistance and recurrence.

1. Introduction

Grade IV astrocytoma (Glioblastoma; GBM) is a classic example of a tumor type whose patients have experienced little improvement in survival prognosis since chemotherapy was introduced as adjuvant therapy to the standard surgery and radiotherapy [1]. Despite highly intense therapeutic regimen including surgical resection, followed by radio and chemotherapy, tumors invariably recur and only few months are added to the patients' life by this treatment [1], leading to a five-year survival rate lower than 5% [2].

Standard GBM chemotherapy employs the alkylating agent temozolomide (TMZ), which acts through the methylation of nucleotide residues in DNA on position *N7* (70%) of guanine, *N3* (9%) of adenine and O^6 of guanine (~6%) [3]. The first two adducts that represent the most frequent changes caused by TMZ, are quickly repaired by the base excision repair (BER) mechanism [4]. On the other hand, methylation of O^6 of guanine is repaired by the specific repair enzyme called O^6-methylguanine-DNA methyltransferase (MGMT) [5], which removes the alkyl groups at O^6-guanine [6], thus directly eliminating this toxic DNA modification. The high expression of this enzyme represents the most common mechanism of resistance to TMZ in GBM cell lines [7]. However, despite the prognostic importance of the methylation status of the MGMT promoter for the response to TMZ, inhibition of MGMT did not significantly increase the survival of recurrent GBM patients treated with TMZ [8].

If not repaired, the methylation on O^6 position leads to a mispairing of O^6-methylguanine with thymidine and futile cycles of DNA repair, through mismatch repair system (MMR). This leads to extensive DNA strand breaks and consequent cellular responses, which include an initial cell cycle arrest [9], as an attempt to provide more time to repair the DNA-damages, followed by

Abbreviations: GBM, glioblastoma; TMZ, temozolomide; CPD, Cumulative Population Doubling; RTCT, Relative Time to Cross a Threshold; rAUC, relative Area Under Curve; RPR, Relative Proliferation Rate; RendCPD, Relative end CPD

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apoptosis [10] or senescence [9], if the damages are not fully repaired [4]. Other mechanisms can be triggered by TMZ in glioblastoma, such as autophagy [11,12], which can interfere in the survival of cells to TMZ. Thus, malfunction of MMR repair system represents a crucial mechanism of resistance to alkylating agents, since this mechanism is responsible for detecting and transmitting the damage signal to downstream cell signaling pathways [13].

Despite this set of classical and well described acute effects produced by TMZ in several GBM cell lines, doses of TMZ found in the plasma of patients almost never completely eliminate all cells in culture and no reports in the literature studied in vitro the surviving subpopulation after the TMZ treatment. Moreover, several works describe the acute effects of TMZ in GBM cells using treatment schedules and dosage of TMZ far from clinical reality, limiting the relevance of this data.

Therefore, in this work we assessed several early cellular responses using therapeutically relevant doses of TMZ, in six GBM cell lines, during 5 days and monitored the remaining cells in the absence of drug for additional 23 days, ending one cycle of 28 days of TMZ treatment, representing the most common schedule used in GBM patients [14]. An extended analysis of cell proliferation revealed long lasting effects of TMZ, and interesting kinetics of regrowth of the surviving population.

2. Material and methods

2.1. Cells and drugs

Human GBM cell lines U87-MG, A172, U251-MG, U138-MG and a murine GBM cell line C6 were obtained from American Tissue Culture Collection (ATCC, Rockville, MD). U343-MGa was gently provided by Dr. Paulo Roberto Dauria Vieira de Godoy (Department of Genetics, University of São Paulo (USP), Brazil) [15]. All culture material and reagents were purchased from Gibco Laboratories (Grand Island, NY, USA). Cells were cultured in DMEM low glucose supplemented with 10% of Fetal Bovine Serum (FBS), 1% penicillin/streptomycin and 0.1% amphotericin B at 37 °C and 5% CO_2 in a humidified incubator. TMZ was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO, Acros Organics, NJ, USA). Cells were treated according to scheme shown in Fig. 1.

2.2. Cell cycle distribution, autophagy and senescence analysis

For cell cycle distribution analysis, cells were seeded at 4×10^4 cells/well in a 12-well plate and treated with TMZ 50 μM for 3 days.

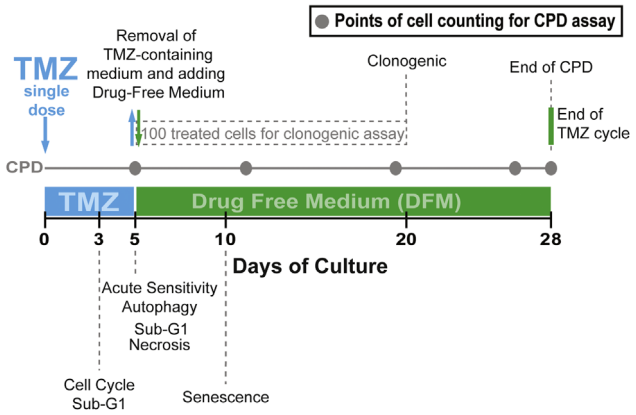

Fig. 1. Experimental design of temozolomide treatment in GBM cells. Schematic figure representing the in vitro GBM treatment regimen established based on standard therapeutic regimen applied in patients with grade IV glioma (GBM). The timeline shows the specific points when all analysis was performed.

After, cells were fixed in ice-cold ethanol 70% (v/v in PBS) for at least 2 h at −20 °C, washed with PBS, and stained with a solution containing 50 μg/mL PI, 0.1% Triton X-100 and 50 μg/mL RNAse for 30 min. Quantification of autophagy levels was done measuring the generation of acidic vacuolar organelles (AVOs), a typical feature of a late phase autophagy [16], through Acridine Orange (AO) staining. This marker produces a red fluorescence in AVOs (mainly late autophagosomes) and a green fluorescence in the rest of the cell. Cells were incubated with AO (2.7 μM) for 15 min at room temperature and quantified no later than 20 min after start of incubation. The above mentioned methods were analyzed with a GUAVA flow cytometer plus GUAVA Cytosoft software.

SA-beta-gal enzyme activity was measured by seeding 10^4 cells/well in a 12-well plate and the assay was performed at day 10 (Fig. 1) as described [17]. Briefly, cells were washed with PBS, fixed with 2% paraformaldehyde for 30 min at room temperature and incubated with fresh SA-beta-gal staining solution (1 mg/mL X-gal (Sigma), 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$) for 8–12 h at 37 °C. Cells were then marked with a solution containing 300 nM DAPI and 0.1% triton X-100 (v/v in PBS) for 15 min at room temperature and micrographed.

2.3. Nuclear morphometric analysis (NMA)

The nuclear morphometric analysis is a technique developed by our group [18] to screen several cell responses to therapy. Nuclei with both large and regular phenotype indicate a senescence state [18]. Cells were seeded and treated as described in SA-beta-gal assay. Five to ten fluorescent micrographs from DAPI stained cells were obtained from each well, followed by quantification of around 500 nuclei using the Software Image Pro Plus 6.0 (IPP6 – Media Cybernetics, Silver Spring, MD) and the percentage of each nuclear phenotype was determined as described, considering only difference in the nuclear area [18].

2.4. Clonogenic assay

Cells were treated accordingly to Fig. 1, using TMZ doses up to 75 μM. On day 5, remaining cells were counted by flow cytometry and 100 cells/well of each tested TMZ dose were seeded in a 6-well plate to evaluate the ability of the remaining cells to form colonies after 5 days of TMZ treatment. On day 20, colonies were fixed with methanol, followed by staining with 0.1% of crystal violet. The number of colonies was counted and their highest diameter was measured using Image Pro Plus 6.0 Software. The relative amount of colonies was used in Fig. 4 considering the control as 100%.

2.5. Acute sensitivity and Cumulative Population Doublings (CPD)

Cells were seeded in 24-well plates, treated and analyzed as indicated in Fig. 1. Human GBM cells were plated at a density of 1.5×10^4 cells/well and murine GBM cells (C6) at density of 0.8×10^4 cell/well and treated. On specific days, a small proportion of cells was used to quantify, by flow cytometry, the amount of remaining cells and a part of the same sample was re-seeded. The acute sensitivity was obtained through the ratio between the absolute amount of cells in the treated group in relation to the control on day 5. EC50 was calculated using excel with ED50 plus v1.0 (INER/México) (n = 3). To obtain the population doubling (*PD*) values, the initial and final values of cells in each time interval was plotted in a formula: $PD = (\ln(FN) - \ln(IN))/\ln 2$, where *FN* (Final Number) corresponds to the number of cells in the well at time of passage and *IN* (Initial Number) is the number of cells seeded at the previous passage [19,20]. The cumulative values of PD were plotted in a graph versus days of culture. The analysis of the CPD