

Original article

Glioblastoma stem cells resistant to temozolomide-induced autophagy

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Keywords: glioblastoma; neoplastic stem cells; chemoresistance; temozolomide

Background Recent studies have demonstrated the existence of a small fraction of cells with features of primitive neural progenitor cells and tumor-initiating function in brain tumors. These cells might represent primary therapeutic target for complete eradication of the tumors. This study aimed to determine the resistant phenotype of glioblastoma stem cells (GSCs) to temozolomide (TMZ) and to explore the possible molecular mechanisms underlying TMZ resistance.

Methods Freshly resected glioblastoma specimen was collected and magnetic isolation of GSCs was carried out using the Miltenyi Biotec CD133 Cell Isolation kit. The cytotoxic effect of TMZ on CD133⁺ and CD133⁻ glioblastoma cells was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Autophagy-related proteins (Beclin-1, LC3 and Atg5) and cleaved caspase-3 (p17) were analyzed by Western blotting. Immunofluorescent staining was used to detect Atg5, glial fibrillary acidic protein (GFAP) and CD133 expression in glioblastoma cells. Statistical analysis was carried out using SPSS 10.0 software. For all tests, the level of statistical significance was set at $P < 0.05$.

Results CD133⁺ glioblastoma cells exhibited neurosphere-like growth *in vitro* and high expression of CD133 stem cell marker. The growth-inhibiting rate in CD133⁻ glioblastoma cells treated with 5 or 50 $\mu\text{mol/L}$ TMZ was significantly higher than that in CD133⁺ glioblastoma cells ((14.36 \pm 3.75)% vs (2.54 \pm 1.36)% or (25.95 \pm 5.25)% vs (2.72 \pm 1.84)%), respectively, $P < 0.05$). Atg5, LC3-II and Beclin-1 levels were significantly lower in CD133⁺ glioblastoma cells than those in autologous CD133⁻ cells after TMZ treatment ($P < 0.05$). Caspase-3 was mildly activated only in CD133⁻ glioblastoma cells after exposure to TMZ ($P < 0.05$). Immunofluorescent staining revealed elevated expression of Atg5 in GFAP⁺ cells following TMZ treatment.

Conclusions The GSCs display strong capability of tumor's resistance to TMZ. This resistance is probably contributed by the CD133⁺ cells with down-regulation of autophagy-related proteins. Future treatment should target this small population of cancer stem cells in tumors to improve survival of patients.

Chin Med J 2009;122(11):1255-1259

Despite progress in study on the molecular aspects of malignant gliomas, the prognosis of these brain tumors continues to be dismal. The median survival of patients with glioblastoma multiforme (GBM), the most aggressive glioma in adults, has remained from 9 to 12 months for decades.¹ Temozolomide (TMZ) was the first new chemotherapeutic agent approved for treatment of high-grade malignant gliomas in more than 20 years. Despite the fact that TMZ contributes significant therapeutic benefits in GBM patients, the addition of TMZ to radiotherapy only resulted in a slightly longer median survival time in newly diagnosed GBM patients, 14.6 versus 12.1 months, and the best 5-year survival of 9.8%.² Recent studies propose the existence of a small fraction of cells with features of primitive neural progenitor cells and tumor-initiating function in brain tumors. These cells, known as cancer stem cells (CSCs) with stem-like properties, constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor.³

Activation of cell death program has been shown to be

responsible for chemotherapy-induced cytotoxicity in tumor cells, while alterations in the death machinery have been related to chemoresistance in gliomas.⁴ However, conventional approaches targeting the overall population of tumor cells may well spare CSCs owing to their idiosyncratic properties.⁵ Several studies indicated that altered expression of apoptosis-related proteins and DNA repair protein O6-methylguanine-DNA-methyltransferase (MGMT) may render glioblastoma stem cells (GSCs) strongly resistant to apoptosis induced by various therapeutic drugs including TMZ.^{5,6} Autophagy, also

DOI: 10.3760/cma.j.issn.0366-6999.2009.11.004

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This study was supported by a grant from National Natural Science Foundation of China (No. 30772551).

know as programmed cell death type II, represents an alternative tumor-suppressing mechanism to overcome, at least partly, the dramatic resistance of many cancers to proapoptotic chemotherapy.⁷ Autophagy, rather than apoptosis, has been associated with TMZ-induced cytotoxicity in glioma cells.⁸ Does this machinery also work on GSCs? This study aimed to explore autophagy-related molecular mechanisms in GSCs in response to TMZ. Our finding suggests that down-regulation of autophagy proteins might be important mechanisms for GSCs to evade TMZ-induced cytotoxicity.

METHODS

Reagents

TMZ was kindly provided by Tasly Pharmaceutical Co. Ltd (China). The following antibodies were used: glial fibrillary acidic protein (GFAP), β -tubulin, cleaved caspase-3 (p17) (Santa Cruz, USA); CD133, Atg5, Beclin-1, LC3B (Cell Signaling, USA); fluorescein isothiocyanate (FITC)- or Cy3-conjugated secondary antibodies (Jackson Laboratory, USA).

Brain tumors specimens, cell sorting and culture

Primary glioblastoma, named GBM-04, was freshly obtained from the operating room following approved protocols and verified by pathologists. CD133⁺ glioblastoma cells were isolated and cultured as described previously by Singh et al.³ Briefly, specimens or xenografts were chopped manually, dissociated with collagenase/dispase, and cultured in stem cell medium (DMEM/F12 medium supplemented with 2% B27 minus vitamin A (Invitrogen, USA), 25 ng/ml of epidermal growth factor (EGF) and 20 ng/ml of basic fibroblast growth factor (bFGF). Within 3 days of primary culture, cells were centrifuged, triturated with a fire-narrowed Pasteur pipette, and resuspended in phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA) and 2 mmol/L ethylenediaminetetraacetic acid (EDTA). Magnetic isolation of GSCs was carried out using the Miltenyi Biotec CD133 Cell Isolation kit (Miltenyi Biotec GmbH, Germany). CD133⁺ and CD133⁻ sorted cell populations were resuspended in stem cell medium.

Cytotoxicity assay

The cytotoxic effect of drugs on glioma cells was determined by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.⁹ Briefly, 1×10^3 cells were plated in 100 μ l of medium in 96-well microtiter plates pre-coated with poly-ornithine and incubated for 24 hours. Drugs were added and the cells incubated for a further 72 hours, dimethyl sulfoxide was used as solvent control. MTT solution was added to each well and incubated at 37°C for 2 hours. The reaction was stopped by adding dimethyl sulfoxide. The amount of MTT formazan product was determined by measuring absorbance with a microplate reader at a test wavelength of 570 nm and a reference

wavelength of 630 nm. Optical density (OD) value was used to express the absorbance of the dual wavelengths. The growth-inhibiting rate of tumor cells equals $(1 - (\text{the OD values of treated groups} / \text{the OD values of control group})) \times 100\%$. Data were the mean of triplicate experiments.

Immunofluorescence staining and microscopy

Cells were cultured on cover slips pre-coated with poly-lysine. After various treatments, cells were fixed in 4% paraformaldehyde, permeated with 0.25% Triton X-100, blocked with 3% normal goat serum, stained with the first antibody overnight, and labeled with a goat anti-mouse or goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) or Cy3. Cells were counterstained with anti-fade sealant containing 4'-diamidino-2-phenylindole (DAPI) (Vectashield, USA) and examined under fluorescence microscope BX61 (Olympus, Japan). Pictures were captured with DP71 CCD digital camera (Olympus).

Western blotting

Cell lysates were prepared with cell lysis buffer (Cell signaling, USA). After sonication, centrifugation, and protein assay (Pierce protein assay kit, USA), 50 μ g protein and an equal volume of $2 \times$ sample buffer (62.5 mmol/L Tris-HCl pH6.8, 2% (w/v) sodium dodecyl sulfate, 10% glycerol, 50 mmol/L dithiothreitol, 0.01% (w/v) bromophenol blue) were heated at 94°C for 5 minutes. Proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transblotted onto a polyvinylidene difluoride (PVDF) transfer membrane (Bio-Rad, USA). The blot was blocked in PBS containing 0.1% Tween-20 and 5% skim milk at 37°C for 1 hour. The membrane was then incubated in primary antibody (1:200) at 4°C overnight, followed by treatment with secondary antibody conjugated with horseradish peroxidase (1:1000). Proteins were visualized using the ECL system (Amersham Biosciences, USA) and quantified using the Image J software version 1.37 from National Institute of Health (MA, USA). Data were the mean of triplicate experiments.

Statistical analysis

Statistical evaluations were carried out using SPSS 10.0 software (SPSS Inc, USA). For all tests, the level of statistical significance was set at $P < 0.05$. The experimental data were expressed as mean \pm standard deviation (SD). Unless otherwise specified, Student's *t* test was used.

RESULTS

Characterization of GSCs

As shown in Figure 1A, HE staining validated GBM04 primary tumor to be glioblastoma multiforme. After CD133 magnetic sorting, sorted GSCs were cultured in stem cell medium. Neurosphere-like gliomaspheres

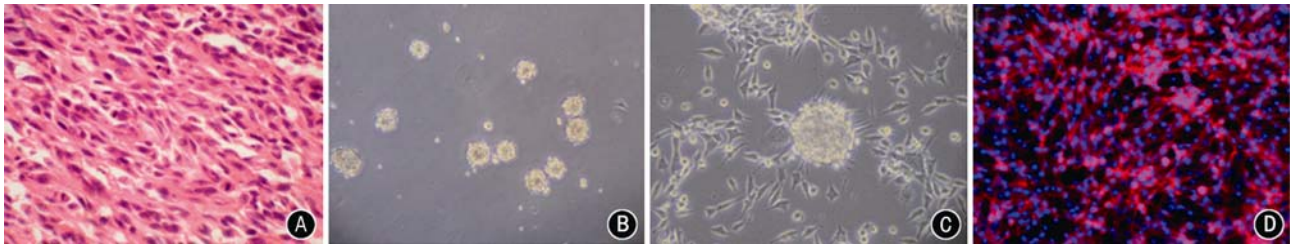


Figure 1. Characterization of CD133⁺ GSCs. **A:** HE staining of GBM04 primary tumor (Original magnification ×400). **B:** After sorting, CD133⁺ GSCs grow as neurosphere-like gliomaspheres in stem cell medium (Original magnification ×40). **C:** CD133⁺ GSCs grow adherent to poly-lysine-coated bottom (Original magnification ×200). **D:** Sorted GSCs exhibit highly expressed CD133 (red), DAPI staining (blue) was performed to identify cells (Original magnification ×200).

appeared after 3-day culture (Figure 1B). GSCs can also grow as an adherent monolayer in poly-ornithine-coated plate (Figure 1C). Immunofluorescence staining showed highly expressed CD133 in GSCs (Figure 1D).

Cytotoxic effects of TMZ on glioma cells

The cytotoxic effects of TMZ on CD133⁺ and CD133⁻ glioma cells are shown in Table 1. The growth-inhibiting rate of CD133⁻ cells after TMZ (5 or 50 μmol/L) treatment was higher than that of CD133⁺ cells (*P* < 0.05).

Table 1. Cytotoxic effects of temozolomide on glioma cells

Groups	Growth inhibiting rate (%)	
	CD133 ⁺ cells	CD133 ⁻ cells
Control (DMSO)	1.52±1.25	2.04±1.56
TMZ (5 μmol/L)	2.54±1.36	14.36±3.75*
TMZ (50 μmol/L)	2.72±1.84	25.95±5.25*

**P* < 0.05, as compared with CD133⁺ cells group (*n* = 3).

Expression of autophagy-related proteins following TMZ treatment

The expressions of autophagy-related proteins Beclin-1, Atg5 and LC3 were identified by Western blotting in groups treated with/without TMZ (50 μmol/L, 72 hours). The results are shown in Figure 2, and the relative OD values determined by Image J software are displayed in Table 2. TMZ treatment caused increased Beclin-1, Atg5, LC3-II and cleaved caspase-3 levels in CD133⁻ cells, but not in CD133⁺ cells (*P* < 0.05). There were no statistical differences of Beclin-1, Atg5, LC3-II and cleaved caspase-3 expression found between the control and TMZ-treated group in CD133⁺ cells (*P* > 0.05). All results suggest CD133⁺ cells are less responsive to TMZ-induced autophagy.

Expression of Atg5 in glioblastoma cells

As shown in Figure 3, expression of Atg5 was detected by immunofluorescence staining. Enormous punctate Atg5 localization emerged after TMZ treatment (50 μmol/L, 72 hours). GFAP⁺ glioblastoma cells displayed high level of Atg5 in both the control and TMZ-treated cells, as compared with that of CD133⁺ cells.

DISCUSSION

In 2004, Singh and colleagues³ successfully isolated CSCs from different types of brain tumors. CSCs were found exclusively in the fraction of cancer cells

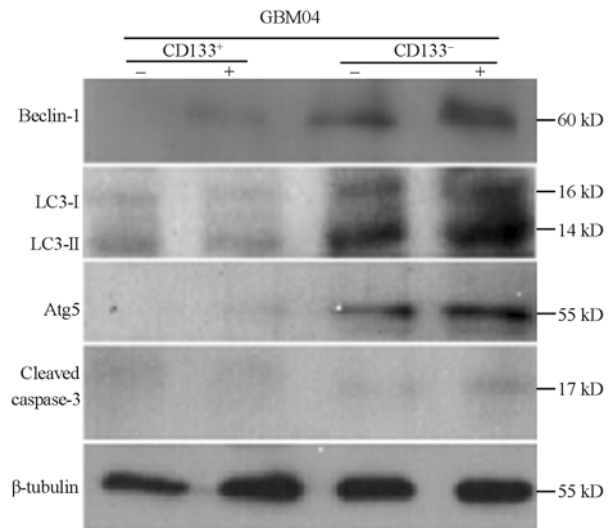


Figure 2. Western blotting analysis for expression of autophagy-related proteins following TMZ treatment. The pictures are scanned and analyzed by Image J software. β-tubulin was used as a loading control.

Table 2. Quantification of autophagy-related proteins following TMZ treatment

Proteins	CD133 ⁺ cells		CD133 ⁻ cells	
	Control	TMZ	Control	TMZ
Beclin-1	0.001±0.001	0.002±0.001 ‡	0.035±0.005	0.048±0.006*†
Atg5	0.001±0.001	0.001±0.001 ‡	0.021±0.004	0.035±0.005*†
LC3-II	0.006±0.002	0.005±0.002 ‡	0.035±0.006	0.049±0.008*†
Cleaved caspase-3	0.001±0.001	0.001±0.001 ‡	0.001±0.001	0.008±0.002*†

**P* < 0.05, compared with the control group in CD133⁻ cells (*n* = 3); †*P* < 0.05, compared with TMZ-treated group in CD133⁺ cells (*n* = 3); ‡*P* > 0.05, compared with the control in CD133⁺ cells (*n* = 3).

expressing CD133. An injection of only 100 CD133⁺ cells into the NOD-SCID mouse brain led to the growth of a tumor that could be serially transplanted and was histologically identical to the tumor harbored by the patient from whom these cells were derived. In contrast, the CD133⁻ tumor cells failed to form tumors, even when 1000-fold more CD133⁻ cells were injected into the brains of the mice, suggesting that the brain tumor stem cells were always in the CD133⁺ population.³ In this study, we successfully enriched CD133⁺ GSCs from clinical specimen.

Activation of the cell death program has been shown to be responsible for chemotherapy-induced cytotoxicity in

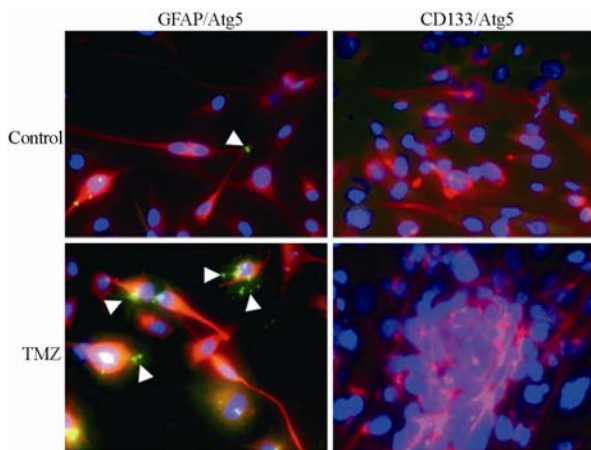


Figure 3. Differential expression of Atg5 in glioblastoma cells following TMZ treatment. GFAP and CD133 were shown as red, while Atg5 as green. Cell nucleus (blue) was counterstained by DAPI. White arrowhead, Atg5-localized autophagosomes (Original magnification $\times 1000$).

tumor cells, while alterations in the death machinery have been related to chemoresistance in gliomas.⁴ Eramo et al¹⁰ suggested that drug resistance observed in GSCs may depend on abnormalities of the cell death pathway such as overexpression of antiapoptotic factors or silencing of key death effectors. A study by Liu and his colleagues⁶ demonstrated for the first time that an increased resistance of CD133⁺ brain tumor stem cells in response to treatment with chemotherapeutic agents including TMZ, compared with autologous CD133⁻ cells. Gene expression studies revealed a higher expression of multi-drug resistance gene BCRP1 and DNA repair genes such as MGMT, as well as genes that inhibited apoptosis in the CD133 expressing CSCs.^{5,6}

Autophagy represents an alternative tumor-suppressing mechanism to overcome, at least partly, the dramatic resistance of many cancers to radiotherapy and proapoptotic related chemotherapy.⁷ Unlike apoptosis, autophagy is a caspase independent process characterized by the accumulation of autophagic vacuoles in the cytoplasm accompanied by extensive degradation of the organelles such as mitochondria, polyribosomes and the endoplasmic reticulum, which precedes the destruction of the nucleus.⁷ Autophagy machinery can be activated by various agents such as DNA damages,¹¹ disruption of PI3K/AKT/mTOR signaling,¹² deprivation of nutrients or amino acids,¹³ and oxidative stress.^{14,15} The components of the molecular machinery responsible for autophagy are products of the autophagy-related (Atg) genes.¹⁶ These genes control a number of aspects of the autophagic process including induction by Beclin-1 (Atg6) and autophagosomal vesicle formation through Atg12-Atg5 and LC3 (Atg8).¹⁷ Additionally, Bcl-2 binds Beclin-1 to inhibit Beclin-1-dependent autophagy, thereby functioning both as a pro-survival and as an anti-autophagic regulator.¹⁸

TMZ contributes significant therapeutic benefits in

glioblastoma patient.^{2,19} Part of TMZ cytotoxic activity is exerted through proautophagic processes, at least in glioblastoma cells, as a result of the formation of O6-methylguanine in DNA, which mispairs with thymine during the following cycle of DNA replication.⁸ Glioma cells thus respond to TMZ by undergoing G2/M arrest, but ultimately die from autophagy.^{20,21} However, Our finding suggested that CD133⁺ GSCs were less susceptible to TMZ-induced autophagy, as compared with CD133⁻ cell fractions. We hypothesized that suppression of autophagy-related proteins might render GSCs strongly resistant to autophagic cell death. Our further study showed autophagy-related proteins such as Beclin-1, Atg5 and LC3 were still low in CD133⁺ cells following TMZ treatment, as compared with CD133⁻ cell fractions. Therefore, the differential response of CD133⁺ and CD133⁻ cells to TMZ-induced autophagy might contribute to the development of TMZ resistance in GSCs and recurrence after TMZ treatment.

Although GSCs might not be susceptible to classical pathways to autophagy. Recently, Jiang et al²² reported the use of an oncolytic adenovirus, delta-24-RGD, to target the abnormal p16INK4/Rb pathway in brain tumor stem cells. Delta-24-RGD induced enormous autophagic cell death both in cell line models *in vitro* and xenografts. Their results show for the first time that brain tumor stem cells are susceptible to adenovirus-mediated cell death via autophagy. Therefore, understanding the mechanisms of autophagy in GSCs may help to address this issue and might contribute to the development of new effective pharmaceutical approaches for the treatment of brain tumors.

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(Received January 4, 2009)

Edited by JI Yuan-yuan