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 µmol/L TMZ was significantly higher than that in CD133+ glioblastoma cells ((14.36±3.75) vs (2.54±1.36) or (25.95±5.25)% vs (2.72±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.
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.\(^1\) Autophagy, rather than apoptosis, has been associated with TMZ-induced cytotoxicity in glioma cells.\(^3\) 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/disparse, and cultured in stem cell medium (DMEM/F12 medium supplemented with 2% B27 minus Biotec GmbH, Germany). CD133\(^+\) and CD133\(^-\) sorted 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, \(1 \times 10^3\) cells were plated in 100 µl 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 \((1-\text{the OD values of treated groups/the OD values of control group})\times100\%\). 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′-6-diamidino-2-phenylindole (DAPI) (Vectorshild, 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 µg protein and an equal volume of 2 × 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 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′-6-diamidino-2-phenylindole (DAPI) (Vectorshild, USA) and examined under fluorescence microscope BX61 (Olympus, Japan). Pictures were captured with DP71 CCD digital camera (Olympus).

**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 \(P \leq 0.05\). The experimental data were expressed as mean ± 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).

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 1. Cytotoxic effects of temozolomide on glioma cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>CD133⁺ cells</th>
<th>CD133⁻ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (DMSO)</td>
<td>1.52±1.25</td>
<td>2.04±1.56</td>
</tr>
<tr>
<td>TMZ (5 µmol/L)</td>
<td>2.54±1.36</td>
<td>14.36±3.75*</td>
</tr>
<tr>
<td>TMZ (50 µmol/L)</td>
<td>2.72±1.84</td>
<td>25.95±5.25*</td>
</tr>
</tbody>
</table>

*P <0.05, 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 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.

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

<table>
<thead>
<tr>
<th>Proteins</th>
<th>CD133⁺ cells</th>
<th>CD133⁻ cells</th>
<th>Control</th>
<th>TMZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beclin-1</td>
<td>0.001±0.001</td>
<td>0.001±0.001</td>
<td>0.035±0.005</td>
<td>0.048±0.006*</td>
</tr>
<tr>
<td>Atg5</td>
<td>0.001±0.001</td>
<td>0.001±0.001</td>
<td>0.021±0.004</td>
<td>0.035±0.005*</td>
</tr>
<tr>
<td>LC3-II</td>
<td>0.066±0.002</td>
<td>0.005±0.002</td>
<td>0.035±0.006</td>
<td>0.049±0.008*</td>
</tr>
<tr>
<td>Cleaved caspase-3</td>
<td>0.001±0.001</td>
<td>0.001±0.001</td>
<td>0.001±0.001</td>
<td>0.008±0.002*</td>
</tr>
</tbody>
</table>

*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).

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 colleagues successfully isolated CSCs from different types of brain tumors. CSCs were found exclusively in the fraction of cancer cells 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. 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
tumor cells, while alterations in the death machinery have been related to chemo-resistance in gliomas. Ermo et al. suggested that drug resistance observed in GSCs may depend on abnormalities of the cell death pathway such as overexpression of anti-apoptotic factors or silencing of key death effectors. A study by Liu and his colleagues demonstrated for the first time that an increased resistance of CD133+ brain tumor stem cells in response to 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.

Autophagy represents an alternative tumor-suppressing mechanism to overcome, at least partly, the dramatic resistance of many cancers to radiotherapy and pro-apoptotic related chemotherapy. 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 mitochondrias, polyribosomes and the endoplasmic reticulum, which precedes the destruction of the nucleus. Autophagy machinery can be activated by various agents such as DNA damages, disruption of PI3K/AKT/mTOR signaling, deprivation of nutrients or amino acids, and oxidative stress. The components of the molecular machinery responsible for autophagy are products of the autophagy-related (Atg) genes. 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). 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.

Although GSCs might not be susceptible to classical pathways to autophagy. Recently, Jiang et al. 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.

REFERENCES

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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 ×1000).
means to combat apoptosis-resistant cancers, with a special emphasis on glioblastomas. Oncologist 2007; 12: 1395-1403.


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