Temozolomide decreases invasion of glioma stem cells by down-regulating TGF-β2

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Abstract. Gliomas are characterized by excessive proliferation, diffuse infiltration and immunosuppression. Recent studies implicate a key role for a restricted population of glioma stem cells (GSCs) in glioma invasive growth and recurrence. Transforming growth factor (TGF)-β2 is a mediator of immunosuppression associated with malignant glioma and also influences pro-invasive functions. Temozolomide (TMZ), is a new alkylating agent with promising antitumour efficacy for malignant gliomas, and the effect of TMZ on GSCs invasion has not been known. To address this issue, we developed studies aimed at neurospheres from primary cultured glioma cells, due to the fact that since neurospheres can be enriched in GSCs, we could examine whether TMZ inhibits the invasion of GSCs. TMZ reduced the TGF-β2-mediated invasion, and down-regulated TGF-β2 expression at the mRNA and protein levels. Thus, these results indicate that TMZ, as a chemotherapeutic agent, can reduce the invasion of GSCs and their immunosuppressive activity. TMZ may be used as an immunomodulating agent for glioma therapy.

Introduction

Glioma is the most prevalent and aggressive type of primary brain tumors in adults and children. This condition is characterized by rapid tumor proliferation, a high level of invasiveness, and suppression of anti-tumor immune surveillance (1). Even though the malignant gliomas are traditionally treated by surgical resection followed by concurrent or sequential local radiotherapy and systemic chemotherapy, high morbidity and mortality persist. Specifically for glioblastoma, the median survival time is 14.6 months, and a 26.5% two-year survival rate has been reported (2). The gliomas are typically comprised of heterogeneous cells, yet not all cells in the same tumor have identical capabilities to proliferate and maintain their growth. Only a subpopulation of tumor cells with stem cell-like properties having the ability of self-renewal and differentiation, manifest such sustained proliferation. Recent evidence indicates that glioma stem cells (GSCs) may give rise to malignant gliomas (3-7). This hypothesis may explain why many traditional treatments fail, possibly due to targeting the wrong domain of cancer cells.

The poor prognosis of malignant gliomas is possibly due to their high invasiveness. Glioma cells that migrate into the surrounding brain parenchyma escape surgical resection and other therapy. Efforts to elucidate the molecular mechanisms responsible for invasion of glioma cells have confirmed that transforming growth factor (TGF)-β2 promotes glioma invasion (8-11). TGF-β2, as one primary mediator of glioma-induced immunosuppression, is tightly linked to tumor progression (12,13). It influences proinvasive fuctions that enable the general spreading of glioma cells by regulating the expression of matrix metalloproteinases (MMPs) (14) and α_V and β3 integrin (8).

Temozolomide (TMZ), a new oral alkylating agent that penetrates the blood-brain barrier and has anticancer activity, is used as a treatment for malignant gliomas. However, it only produces modest benefit as a supplement to radiotherapy (2,15). The highly invasive characteristic of GSCs could explain the failure of treatment for malignant glioma.

This prompted us to investigate the effect of TMZ on GSCs. Here, utilizing GSCs, which have the characteristics of primitive neural progenitor cells and tumor-initiating capacity, we conducted this study to investigate whether TMZ could inhibit TGF-β2-induced invasiveness.

Materials and methods

Cell culture. Tumor samples were obtained from the Department of Neurosurgery in the First Affiliated Hospital of China Medical University. Tumors were classified in accordance with World Health Organization criteria (16). The study was approved by the Research Review Boards of the First Affiliated Hospital of China Medical University. The fresh samples were washed, minced in phosphate-buffered saline (PBS), subjected to enzymatic dissociation before passage through a series of cell strainers, and recovered by centrifugation at 800 x g for 5 min. Tumor cells were cultured in Dulbecco's modified Eagle's

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Table I. Oligonucleotides for real-time RT-PCR.

mRNA	Oligonucleotides	Product size (bp)
TGF-β2	F: GCTTTGGATGCGGCCTATTG R: CCAGCACAGAAGTTGGCATTGTA	137
GAPDH	F: GCACCGTCAAGGCTGAGAAC R: TGGTGAAGACGCCAGTGGA	138

medium (DMEM)/F12 (Gibco, Invitrogen Corp., NY, USA), supplemented with 10% fetal bovine serum (FBS, Hyclone, UT, USA). The procedures were performed as described, with some modification (3-7,17). Briefly, after the monolayer cultures, primary cultured glioma cells (PCGCs) were switched into serum-free DMEM/F12 containing 20 ng/ml human recombinant epidermal growth factor (EGF, Invitrogen, CA, USA), 20 ng/ml human recombinant basic fibroblast growth factor (bFGF, Invitrogen), B27 (1:50, Invitrogen), and were fed by replacing half of the medium every 3 days. All cells grew at 37˚C in an atmosphere of 5% CO_2 and 95% room air.

Immunocytochemistry staining of neurospheres and the differentiated glioma cells. Briefly, neurospheres were seeded in poly-L-lysine-coated glass coverslips and allowed to adhere overnight. In the differentiation assays, the neurospheres were cultured in DMEM/F12 supplemented with 10% FBS for 7 days. Then all the cells were fixed using 4% paraformaldehyde at room temperature for 30 min, and washed three times with PBS. Next, the cells were permeabilized with 0.3% Triton X-100 (Sinopharm Corp., Shanghai, China) for 30 min, washed with PBS, and incubated in 5% bovine serum albumin (BSA, Zhongshan Goldenbridge Biotechnology Corp., Beijing, China) for 20 min. After removal of the blocking solution, the cells were incubated at 4˚C overnight in 1:200 rabbit anti-human GFAP, a glial fibrillary acidic protein (Chemicon International, Temecula, CA, USA), 1:200 mouse anti-human β-III-tubulin (Millipore, MA, USA), or 1:200 mouse anti-human CD133 (Abcam, Cambridge, UK). After washing with PBS, cells were incubated at 37˚C for 2 h with 1:250 sheep anti-mouse IgG Cy3 conjugate (Sigma-Aldrich, Inc., NY, USA) or 1:100 goat anti-rabbit IgG FITC conjugate (Sigma-Aldrich). After washing with PBS, the cells were stained with Hoechst 33258 (Sigma-Aldrich) for 5 min to visualize the cell nuclei, and examined under a fluorescence microscope. In control samples, the primary antibody was replaced by isotype IgG. Triton X-100 treatment was omitted when immunostaining for CD133.

Chemotherapeutic agents. Temozolomide was kindly supplied by the Tasly Pharmaceutical Co., Ltd., (China) and was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at 150 mM stock solution. Aliquots of the stock solution were stored at -80˚C and diluted with respective cell-permissive medium.

Real-time reverse transcription polymerase chain reaction (RT-PCR). All the cells, treated with three different concentrations (5, 50, 500 μM) of TMZ for different times (6, 12, 24 and 48 h), were harvested and subjected to total RNA extraction with RNAprep Pure Cell/Bacteria kit (Tiangen Biotech Co., Ltd., Beijing, China) and reverse transcribed by using a Quantscript RT kit (Tiangen Biotech Co., Ltd.), according to the manufacturer's protocol. Real-time RT-PCR was performed in three replicates of each sample using a total volume of 50 μl, reaction mixture that contained 4 μl cDNA as template, each specific primer pairs (Table I) and SYBR® Premix Ex Taq™ II (Takara Biotechnology Corp., Dalian, China). After 30 sec at 95˚C, and 40 cycles of 5 sec at 95˚C and 31 sec at 60˚C were run. GAPDH in each sample served as an endogenous control. The reaction was carried out with ABI Prism 7000 sequence detection system. The fluorescent data were analyzed by relative quantification and the standard curve method.

Enzyme-linked immunosorbent assay (ELISA). TGF-β2 concentrations in cell culture media were measured using commercial ELISA kits (R&D Systems, MN, USA) according to the manufacturer's protocol. Briefly, cells were cultured in the serum-free DMEM/F12 medium (SFM) or treated with TMZ (50 μM) for 48 h, and culture supernatants were harvested and clarified by centrifugation at 800 x g for 5 min before ELISA analysis. Cell numbers in each culture were counted: $1x10^6$ cells were defined as one unit and levels of TGF-β2 were expressed as μg/ml/unit. ELISA analysis was carried out on 96-well microplates pre-coated with the appropriate antibodies. In the next step, 200 μl of serial dilutions of the reference standards or samples were added in each well. After repeating incubation and washing, bound antigen was detected using a conjugated secondary antibody and substrate solutions; OD values at 450 nm were determined using a microplate reader. Each assay was performed in triplicate and the concentrations of TGF-β2 were determined by reference to standard curves.

Cell invasion assay. For this assay, cultured cells used were below 80% confluence, the medium was replaced with SFM and the cells were cultured in an incubator for 24 h. Matrigel Basement membrane matrix (BD Biosciences, MA, USA) diluted by the respective medium was coated in the upper chambers of the Transwell assay (6.5 mm diameter 8.0 μm pore size polycarbonate filters, Costar, Corning, NY, USA), and the plates were incubated overnight in 5% CO_2 incubator at 37˚C. The cells (20,000) were added to the upper chambers. In the experiments with TMZ (50 μM), TGF-β2 antibody (2 μg/ml, Abcam), TGF-β2 (0.4, 2 ng/ml, R&D Systems), or TMZ- or TGF-β2 (2 μg/ml) antibody-pretreated supernatants of GSCs or respective SFM control were added to conditioned media 30 min before addition of cells. Receiver wells were set

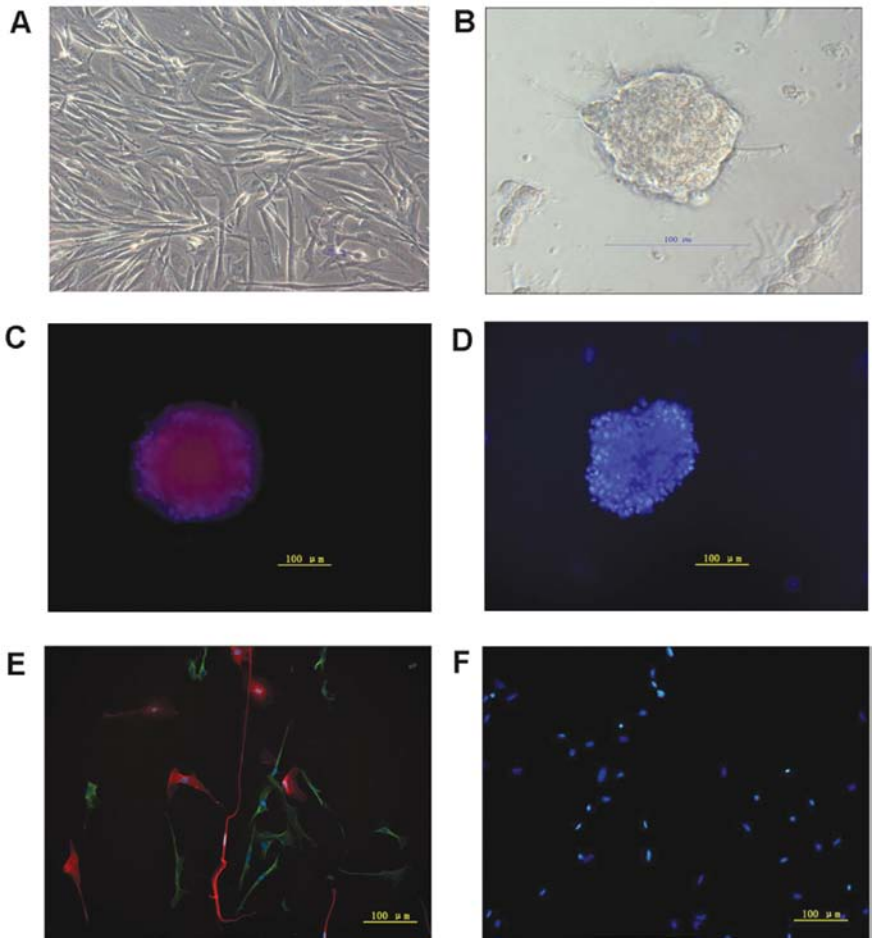

Figure 1. Primary cultured neurospheres and the differentiation of GSCs. (A) Fresh samples were cultured in DMEM/F12, supplemented with 10% FBS. (B) Primary neurospheres were developed from monolayer tumor cells in EGF, bFGF and B27 without serum. (C) Immunocytochemistry was performed on GSCs using CD133. Undifferentiated primary neurospheres expressed CD133 (red) in serum-free medium, and the cell nuclei were stained with Hoechst 33258 (blue). (D) Neurospheres stained with secondary antibody and Hoechst 33258, but with isotype primary antibody (control). (E) The neurospheres cultured in 10% FBS DMEM/F12 medium can give rise to glia and neuronal cells, and these cells were positive for glial (GFAP, green) and neuronal markers (β-III-tubulin, red) (blue, Hochest 33258). (F) Differentiated glioma cells stained with secondary antibody and Hochest 33258, but without anti-GFAP and anti-β-III-tubulin primary antibody (control).

up with respective medium with 20% FBS. Cells were allowed to invade for 24 h, then fixed by methanol and cold acetic acid, then stained by Giemsa (Sigma, St. Louis, USA). All experiments were conducted in triplicate. Nuclei of invasive cells were counted in five high-power fields (x200) and the values were expressed as the mean ± SE.

Statistical analysis. Statistics were generated for all quantitative data with presentation of means ± SE. The significance was determined by t-test using SPSS 10.0 statistical software.

Results

The morphology of neurospheres from PCGCs is similar to cancer stem cells (CSCs). In this study, we used four different GSC lines from PCGSs, GSCs-No.9 and GSCs-No.11 from patients with glioblastoma (Grade IV), GSCs-No.2 and GSCs-No.10 from patients with anaplastic oligodendroglioma (Grade III). PCGCs from fresh clinical samples were first cultured as adherent monolayers (Fig. 1A). To investigate if GSCs could be obtained from PCGCs, we cultured them in serum-free medium in the presence of EGF, bFGF and B27. After 5 days, cells cultured in the EGF-, bFGF- and B27-supplemented serum-free medium readily formed non-adherent, multicellular neurospheres (Fig. 1B), and their growth was steady.

Neurospheres express the marker of CSCs and have the ability to multi-differentiate. Immunocytochemistry was performed on neurospheres using the brain tumor stem cell marker CD133. As shown in Fig. 1C, neurospheres express CD133, which indicated that the majority of cells in the glioma-derived neurospheres are GSCs. As in glioma neurospheres, serum induced the attachment and loss of spherical morphology of neurospheres, decreased the expression of the brain tumor stem cell marker (CD133), and promoted the appearance of differentiation markers (GFAP and β-III-tubulin). In the next step, experiments were carried out to investigate if neurospheres could differentiate in the serum medium with the aid of immunofluorescence. The neurospheres cultured in 10% FBS DMEM/F12 medium can give rise to glia and neuronal cells, and these cells were positive for glial (GFAP)

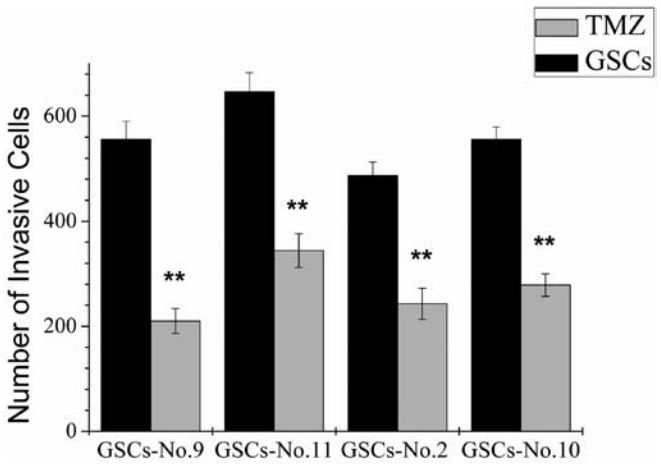

Figure 2. TMZ inhibits the invasion of GSCs. The cells were plated in the upper chambers of a Transwell assay with TMZ (50 μM). Invaded cells were counted in five random fields (mean ± SEM; $^{**}p<0.01$; t-test).

and neuronal markers (β-III-tubulin) (Fig. 1E). These observations indicate that neurospheres obtained from patient-derived glioblastoma multiforme are enriched for glioma stem cells, and are consistent with previous reports (3,18,19) in which CD133-positive GSCs were able to be multipotent and were found to differentiate into glias and neurons.

TMZ decreases GSCs invasion. In order to study the effect of TMZ on GSCs invasiveness, the number of GSCs in response to 20% FBS medium was measured in Transwell chamber. The cells were cultured with SFM alone or TMZ in the upper chambers for 24 h and then stained by Giemsa. The patient-derived GSC lines from four different glioma patients exhibited a significant inhibition of invasion in response to TMZ (Fig. 2). The decreased cell numbers seen in the assay were not due to the effect of proliferation, because TMZ had no effects on cell viability at 24 h (data not shown).

TMZ reduces TGF-β2 expression. Since TGF-β2 is an important immunosuppressive cytokine and represents a pro-invasive factor implicated in promoting glioma cell invasion (20), we next evaluated a possible interaction of TMZ and TGF-β2 in GSCs. The real-time RT-PCR assay showed that the expression of TGF-β2 was stronger in control group cells than in TMZ-treated cells, and TMZ down-regulated TGF-β2 mRNA in a concentration-dependent manner (Fig. 3). For the time-point assay, cells were treated with TMZ (50 μM) for 6, 12, 24 and 48 h (Fig. 4). The TMZ-mediated induction of TGF-β2 mRNA expression was sustained at least for 48 h. These results indicate that the reduction of TGF-β2 mRNA expression by TMZ is a common phenomenon that takes place in GSCs. As expected, the reduction of the TGF-β2 transcript by TMZ (50 μM) resulted in a decrease in TGF-β2 protein secretion as measured by ELISA (Fig. 5).

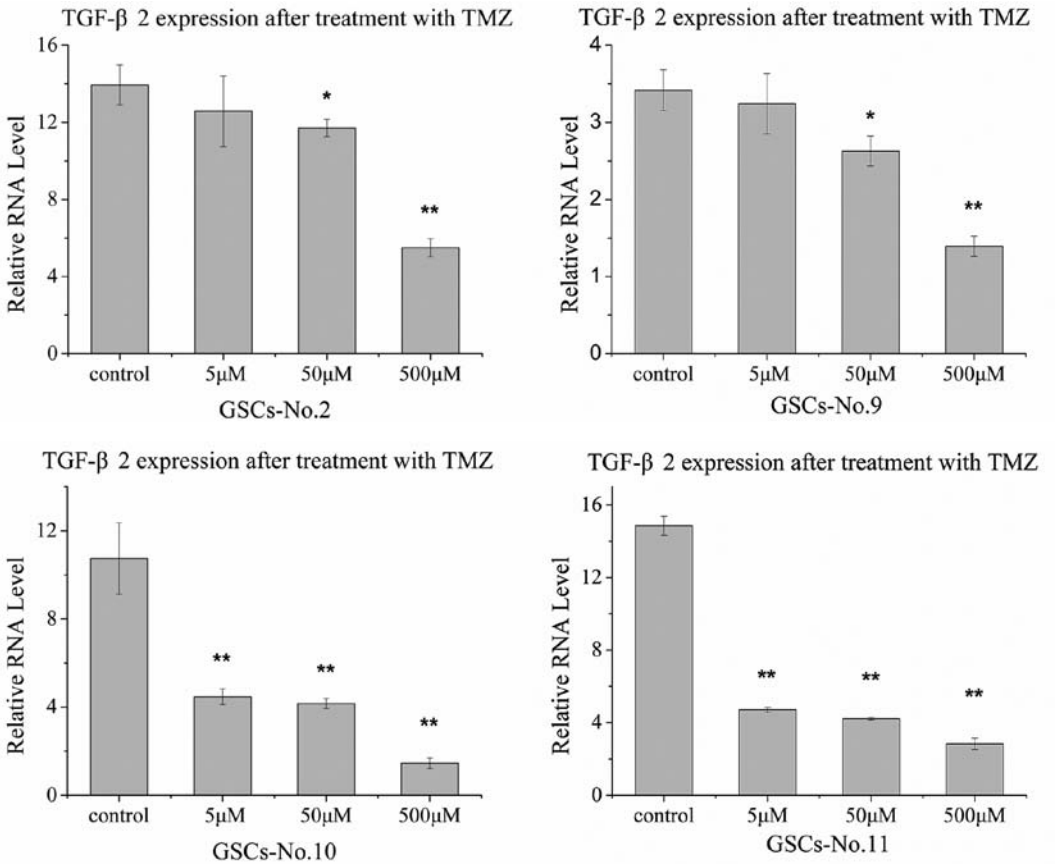

Figure 3. TMZ reduces the expression of TGF-β2 in GSCs at different concentrations. The mRNA expression of TGF-β2 was reduced by TMZ in a concentration-dependent manner as analyzed by real-time RT-PCR in GSCs. GAPDH served as an endogenous control. ($^{*}p<0.05$, $^{**}p<0.01$, significant against the relative RNA level of the control group).

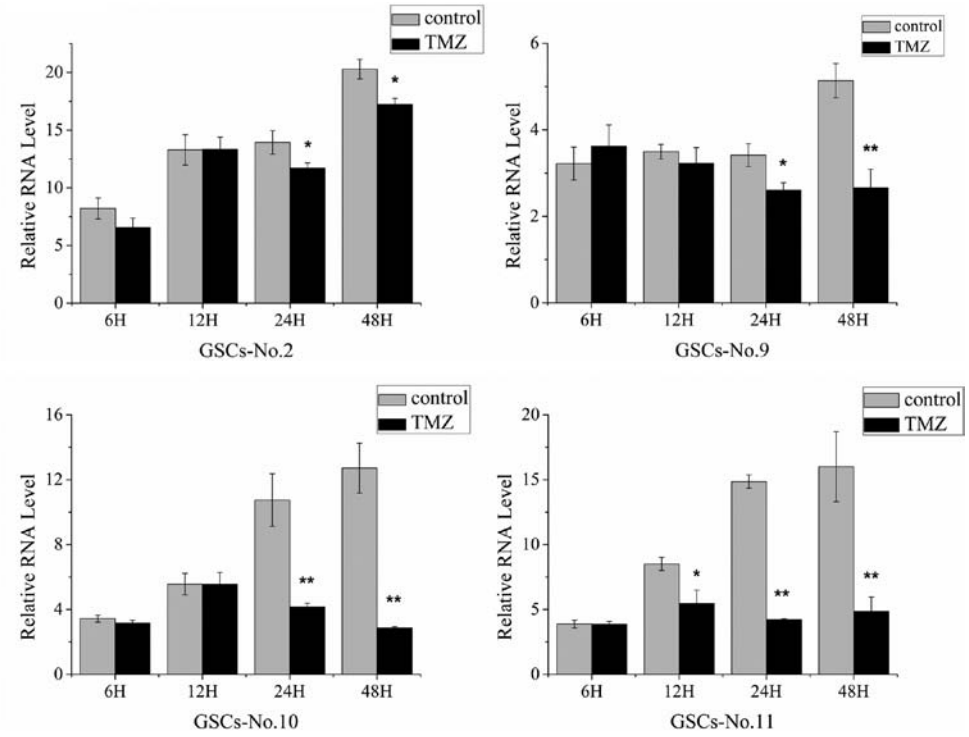

Figure 4. TGF-β2 expression in TMZ (50 μM)-treated cells at 6, 12, 24, and 48 h, respectively (*p<0.05, **p<0.01, significant against the relative RNA level of control group).

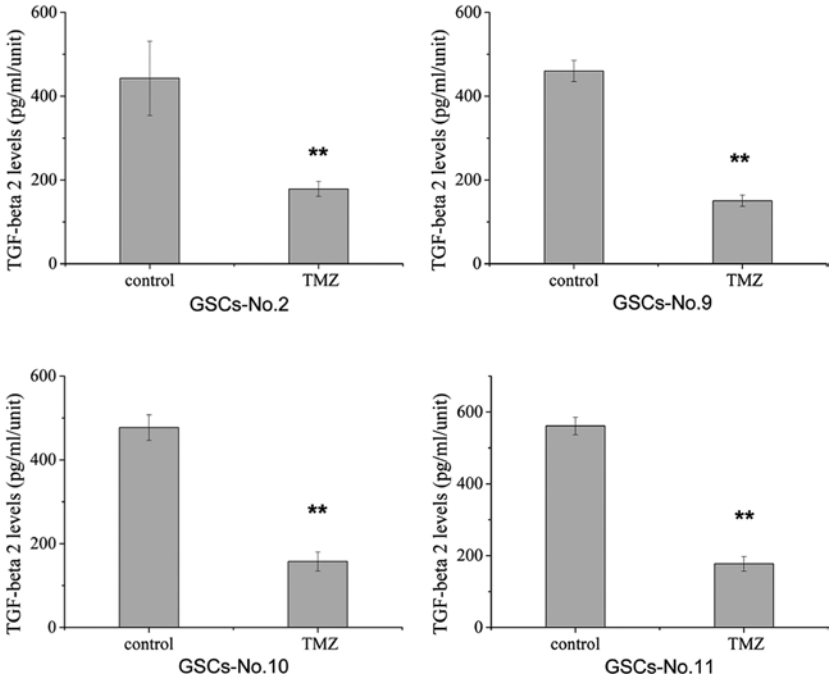

Figure 5. ELISA analysis was used to detect the levels of TGF-β2 in supernatants of control cultured glioma cells or treated with TMZ for 24 h; **p<0.01.

TGF-β2 enhances GCSs invasion. To determine the effect of TGF-β2 on GSCs invasion, GSCs-No.2 and GSCs-No.11 were measured by a Transwell assay. Addition of TGF-β2 for 24 h, increased the number of invaded GSCs (Fig. 6). Moreover, there was a dose-dependent effect of TGF-β2 on the number of neurospheres. These results showed that exogenous TGF-β2 could enhance GSCs invasion.

Reduction of invasion by TMZ through TGF-β2. Since TMZ inhibits the invasion and reduces TGF-β2 expression in GSCs, we further examined the role of TGF-β2 in the biological effects of TMZ on GSCs. Consistent with the altered invasion of GSCs treated with TMZ, a neutralizing TGF-β2 antibody markedly reduced invasion of non-treated glioma cells, but did not affect the residual invasion of glioma cells treated

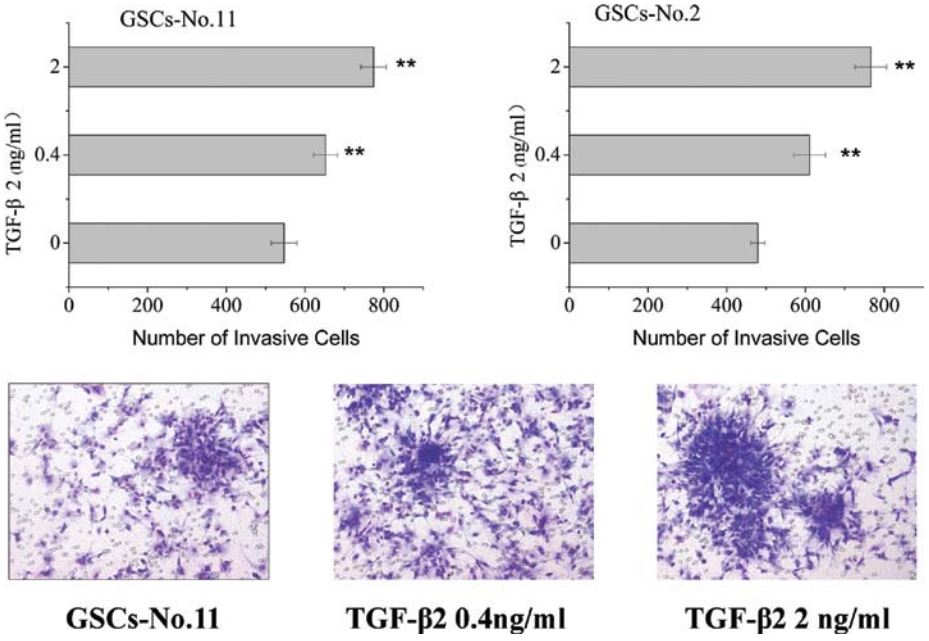

Figure 6. TGF-β2 promotes GSC invasion. Cells were added with medium alone or medium containing TGF-β2 at 0.4 or 2 ng/ml. The number of cells invaded through the membrane was quantified by counting stained cells in 5 random fields of the membrane (original magnification, x200). Data are expressed as the mean ± SEM (*p<0.01).

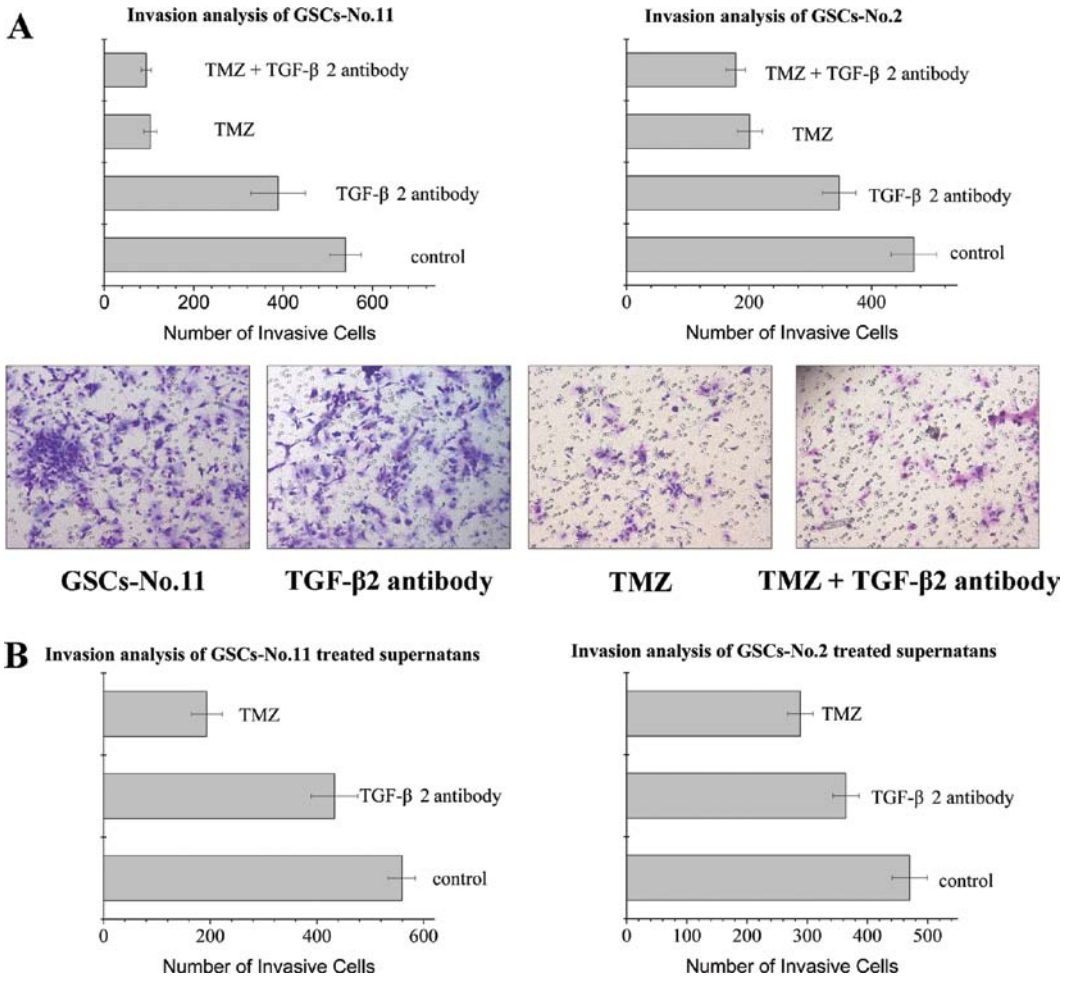

Figure 7. TMZ and TGF-β2 antibody decreases GSC invasion. (A) The cells were plated in the upper chambers of a Transwell assay with TMZ (50 μM), TGF-β2 antibody (2 μg/ml) or control medium. Invaded cells were counted in five random. (B) TMZ-pretreated or TGF-β2 antibody-pretreated supernatants were plated in the upper chambers with cells. The cells invaded through the membrane were counted in five random fields. (mean ± SEM; p<0.05 was defined as significant; t-test).

by TMZ (Fig. 7A). Furthermore, to further substantiate the link between TMZ and TGF-β2, we used supernatants of the GSCs lines treated with TMZ. TMZ-pretreated or TGF-β2 antibody-pretreated supernatants were able to reduce the invasion significantly (Fig. 7B) suggesting that impaired TGF-β2 activity results in the down-regulation of TGF-β2 (Fig. 3). These experiments demonstrated that TMZ mediated the inhibition of invasion in GSCs by reducing TGF-β2 synthesis.

Discussion

To the best of our knowledge, the data presented in this study represents the first report on the action of TMZ on the invasion of GSCs, as well as on the pro-invasion and immune gene of GSCs *in vitro*. We demonstrate that TMZ is a potent invasiveness inhibitor of GSCs (Fig. 2). The MTT assay was carried out after 24 h of incubation with TMZ to examine whether this effect is due to cell proliferation (data not shown). These short incubations had no effect on cell growth. Combined with the data presented in Figs. 2, 3 and 4, this observation suggests that TMZ probably inhibits invasion of GSCs through reducing TGF-β2 mRNA expression. This finding was further confirmed at the protein level through ELISA analyses in GSCs culture supernatants (Fig. 5).

Excessive proliferation, diffuse infiltration of normal parenchyma, and suppression of anti-tumor immune surveillance are key biological features of gliomas. Although surgical resection can relieve the main mass of glioma, adjuvant cellular therapy, or activation of immune cells, are needed to target the glioma cells that infiltrate through normal brain tissue (21). The GSCs are a heterogeneous group of undifferentiated glioma cells defined by their ability for self-renewal, multipotency, and ability to differentiate into progenitor-like glioma cells. GSCs are regarded as being the source of glioma cells and, as with other tumor progenitor cells, express high levels of the immunosuppressive factor, TGF-β2 (22). Meanwhile, GSCs have an additional characteristic to escape from conventional therapies-migration (23). They have a more aggressive invasion than FBS-cultured glioma cells (24,25), therefore, GSCs are considered to be critical therapeutic targets (26,27). TMZ has been considered as a chemotherapeutic agent in high-grade gliomas (2). It can inhibit the proliferation of GSCs if supplied at optimized dosing schemes (28).

In the present study, we examined the role of TMZ in the invasive property of human GSCs. Using four different human primary cultured GSCs lines, we show that TMZ could inhibit the invasion of GSCs (Fig. 2). The next phase of the study was to elucidate the molecular players involved in the invasive inhibition of TMZ on GSCs. TGF-β2 is a cytokine that is released by glioma cells and has been considered central to the progression of glioma and to the immune dysfunction in glioblastoma patients (1). It can promote tumor angiogenesis, enhance invasion and inhibit T cell-mediated immune responses (29). Bruna *et al* observed previously that tumors with high levels of TGF-β2 tend to be more aggressive (30). Platten *et al* found that TGF-β2 promote migration of glioma cells by inducing α_V and β3 integrin mRNA expression and enhancing cell surface expression of α_V and β3 integrin (8). It was previously reported by Wick *et al* (9), that TGF-β2 can control glioma cell invasion via MMP-2. Penuelas *et al* found that TGF-β2 induced the self-renewal capacity and prevented the differentiation of GSCs (24), therefore, TGF-β2 could act on the malignant phenotype of gliomas including invasiveness and immunosuppression and have a important role in the regulation of GSCs. We hypothesized that TMZ inhibited GSCs invasion by altering the expression of TGF-β2. In fact, there was a reduction of TGF-β2 mRNA expression in TMZ-treated GSCs (Figs. 3 and 4). We further confirmed that TMZ-treated GSCs release much less TGF-β2 into the cell culture supernatant than control cells at the protein level using ELISA (Fig. 5).

Since TGF-β2 plays a central role in TMZ-mediated inhibition of GSC invasion, we went on to examine the hypothesis that TMZ affects the biological activity of invasion through TGF-β2. We observed that TGF-β2 could enhance the ability of GSC invasion by the Transwell assay (Fig. 6). Consistent with a key role of TGF-β2 loss in the TMZ-impaired invasion, a neutralizing TGF-β2 antibody inhibits invasion in control cells, but not in TMZ-treated cells (Fig. 7A), which suggests that the residual endogenous TGF-β2 released by TMZ-treated cells plays no role in the residual invasive potential of these cells. To further substantiate the link between TMZ and TGF-β2, we used supernatants of the TMZ-treated GSC cell lines. TMZ-pretreated or TGF-β2 antibody-pretreated supernatants were able to significantly reduce the invasion (Fig. 7B). These observations confirm that the impaired activity of TGF-β2 is a necessary and sufficient consequence of TMZ-treated cells, which could elucidate the phenomenon of inhibited invasion.

In summary, we show for the first time that human GSCs were sensitive to TMZ-mediated invasion. In addition, TMZ altered the invasion of GSCs, most likely mediated by changing TGF-β2 expression. As a result, TMZ could not only reduce the invasion of GSCs, but also decrease TGF-β2 expression. The results have implications for immunotherapy of GBM patients and indicate that TMZ can inhibit GSC invasion and, as an immunomodulating agent, can efficiently rescue the immunogenicity of the targeted GSCs.

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