Journal of Clinical Neuroscience 41 (2017) 144–149

Contents lists available at ScienceDirect

Journal of Clinical Neuroscience

journal homepage: www.elsevier.com/locate/jocn

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Temozolomide does not influence the transcription or activity of matrix metalloproteinases 9 and 2 in glioma cell lines

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ARTICLE INFO

Article history:
Received 11 November 2016
Accepted 13 March 2017

Keywords:
Matrix metalloproteinase 2
Matrix metalloproteinase 9
Temozolomide
Glioblastoma
Semi-quantitative reverse transcription-PCR

ABSTRACT

Glioblastoma multiforme (GBM) is a treatment-resistant malignancy with poor prognosis. Temozolomide (TMZ) is widely used as a first-line drug for GBM. Although this improves patient prognosis, it does not completely eradicate the tumour. Even after total surgical resection, GBM can exhibit uncontrollable invasiveness at the tumour margins owing to activation of matrix metalloproteinases (MMPs) such as MMP-2 and -9; these degrade collagen IV in the basement membrane, which normally prevents cancer invasion. TMZ induces DNA damage and activates transcription factors including c-jun, c-fos, nuclear factor-κβ, and early growth response protein-1, which have putative binding sites on the *MMP-9* promoter. TMZ may therefore enhance tumour invasion by stimulating MMP-9 transcription and enzymatic activity. To test this hypothesis, we investigated *MMP-2* and *-9* mRNA transcription and activity in GBM cell lines treated with TMZ. Human A172 GBM cells were exposed to TMZ (25% and 50% inhibitory concentrations) for 24 or 48 h; cell cycle distribution and mRNA levels of *MMP-2* and *-9* were evaluated using flow cytometry and semi-quantitative reverse transcription PCR, respectively. MMP-2 and -9 enzymatic activities were assessed using gelatin zymography in human A172 and U373 MG GBM cells exposed to TMZ under the same conditions. TMZ altered A172 cell cycle distribution, but not *MMP-2* or *-9* mRNA levels. TMZ did not affect MMP-2 or -9 enzymatic activities in A172 or U373 MG cells. These findings indicated that TMZ is therefore unlikely to promote GBM invasiveness.

1. Introduction

Gliomas are common malignant brain tumours, among which glioblastoma multiforme (GBM) has the worst prognosis [1]. GBM is treated by surgical resection, along with radiotherapy and some types of chemotherapy, but this does not greatly prolong median survival [2]. Temozolomide (TMZ), an orally bioavailable alkylator [3], increases the median survival to 14.6 months [4] and is widely used as a first-line drug for GBM treatment.

TMZ is a small molecule (194 kDa) that has lipophilic properties and shows good biodistribution, including high penetration through the blood–brain barrier into the central nervous system [5,6]. The cytotoxicity of TMZ is attributed to methylation of the O6 position of guanine [7,8], induction of DNA double strand breakage, and tumour cell death by apoptosis [9]. This process does not depend on cell cycling—that is, it affects cells in mitosis and also those in G0/G1 phase (about 50–90% of any proliferative cell population) [10]. However, since the introduction of TMZ as a standard chemotherapy in the U.S., the median survival has remained well below 1 year [11]. One reason for this poor prognosis is the development of therapeutic resistance mediated by the DNA repair protein, O6-methylguanine-DNA methyltransferase, which suppresses the effects of TMZ [12]. Another reason for the poor prognosis is a failure to control the invasiveness of GBM tumour cells, which can infiltrate into the contralateral brain hemisphere or resection plane, even after gross total resection [13,14].

Tumour invasion involves three steps, namely tumour attachment via cell surface receptors that specifically bind to matrix components such as lamin and fibronectin; tumour cell secretion of hydrolytic enzymes that degrade the local extracellular matrix (ECM); and tumour cell locomotion into the proteolytically modified ECM [15]. Malignant brain tumour invasion is accompanied by remodelling of the vasculature and destruction of the ECM in the adjacent normal tissue [16]. Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade ECM components and thus promote tumour invasiveness [17,18]. In particular, the MMP-2 and -9 gelatinases enable cancer cells to degrade collage IV in

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http://dx.doi.org/10.1016/j.jocn.2017.03.048
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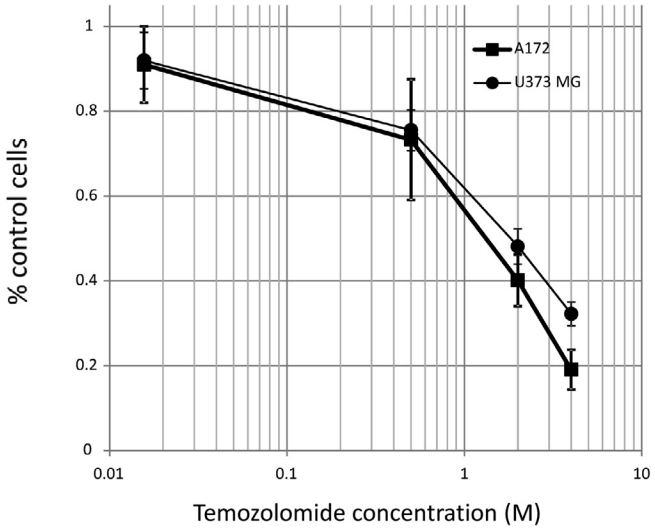

Fig. 1. Cytotoxicity of TMZ in A172 (■) and U373 MG (●) cells. Cells were exposed to the indicated concentrations of TMZ for 48 h and then fixed and stained with Methylene Blue; the absorbance at 595 nm was measured and expressed as a percentage of the level observed in cells cultured without TMZ, which was set as 1. Results represent the mean ± SD of eight experiments.

the basement membrane, a process that is essential for ECM invasion [19,20]. MMP-2 and -9 thus play important roles in glioma invasion and are associated with glioma malignancy [16,21–25]. In the present study, we hypothesised that MMP-2 and -9 could serve as markers of tumour invasiveness.

The DNA damage caused by TMZ induces cellular stress, which can activate transcription factors such as c-jun, c-fos, nuclear factor (NF)-κβ and early growth response protein (egr)-1 [9,26–30]. These transcription factors can bind to the *MMP-9* promoter and induce gene transcription [31,32]; the resultant increase in the MMP-9 protein level is directly associated with its enzymatic activity [33,34]. However, the relationship between TMZ exposure and the transcription and activities of MMP-9 and -2 is unclear. We speculated that TMZ would enhance *MMP-9* transcription and thus modify glioma invasiveness. To test this hypothesis, we examined the transcription and enzymatic activities of MMP-2 and -9 in GBM cells exposed to TMZ.

2. Materials and methods

2.1. Cell lines and TMZ

A172 and U373 MG human glioblastoma cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Cat. No. 12800-082) supplemented with 10% foetal bovine serum in a culture flask until the start of the experiments. TMZ was obtained from Merck (Kenilworth, NJ, USA).

2.2. TMZ cytotoxicity assay

A total of 10^4 dispersed A172 or U373 MG glioma cells were allowed to attach to the bottom of the culture flask for 24 h prior to treatment. Cells were seeded in 96-well plates and exposed to a range of TMZ concentrations (16 μM–4 mM); TMZ was dissolved in distilled water and diluted 1:1 in 2 × DMEM to achieve these final concentrations. After 72 h in culture, the cells were fixed with 5% glutaraldehyde and stained with 0.01% Methylene Blue. The plates were washed prior to eluting the dye using 0.3 N hydrochloric acid. The absorbance of the eluate was measured at 595 nm on a microplate reader (Bio-Rad, Hercules, CA, USA) and used to calculate the 25% and 50% inhibitory concentrations (IC25 and IC50, respectively).

2.3. Cell treatment by TMZ

Cells were exposed to no TMZ or the IC25 or IC50 for TMZ for 0, 24 and 48 h before the assays were carried out. Twofold the required TMZ concentrations were prepared in distilled water and then diluted 1:1 in 2× DMEM. Cells were cultured in 2 ml of this liquid per 25 cm^2 flask. For semi-quantitative reverse transcription (RT)-PCR, cells were exposed to the TMZ IC50 for 48 h.

2.4. Cell cycle analysis

Cells were dispersed with trypsin, resuspended in phosphate-buffered saline, fixed with 100% ethanol, and stained with 0.05 μM propidium iodide with 180 U of RNase for 30 min. Cell cycle distribution was evaluated using flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA). The cell populations in each phase of the cell cycle were analysed manually.

2.5. Semi-quantitative RT-PCR

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Tokyo, Japan), according to the manufacturer's instructions. After treatment with RNase inhibitor and RNase-free recombinant DNase I (Takara Bio, Otsu, Japan) for 30 min, total RNA was reverse transcribed using the PrimeScript RT Master Mix (Takara Bio). The resultant cDNA was used as a template for semi-quantitative RT-PCR with the following primers: *MMP-2*, 5′ TTCTTGTCGCGGTCGTAGTC and 3′ ACAGCCCTGCAAGTTTCCAT; *MMP-9*, 5′ CTTCTACGGCCACTACTGTG and 3′ GCCAGTACTTCCCATCCTTG; and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), 5′ CCCAGCAAGAGCACAAGAG and 3′ CCTCTTCAAGGGGTCTACATG. The reactions were carried out on an ABI 7300 system (Applied Biosystems, Foster City, CA, USA) at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s (CYBR Premix Ex Taq II; Takara Bio). The specificity of the amplification was confirmed by melting curve analysis, and *MMP-2* and *-9* transcript levels were compared to that of the internal control (*GAPDH*) using the relative quantification (ΔΔCt) method [35].

2.6. Gelatin zymography

Cell culture medium conditioned by treated A172 and U373 MG cells was collected. Sodium dodecyl sulphate (SDS) polyacrylamide (7.5%) was co-polymerised with 2.5 mg/ml gelatin (Nitta Gelatin, Osaka, Japan) and the gels were loaded with 10 μl gel-loading buffer [62.5 mM Tris–HCl (pH 6.8), 20% glycerol, 10% SDS, and 0.1% Bromophenol Blue] and the same volume of sample. These were separated by SDS polyacrylamide gel electrophoresis. The gel was washed with denaturation buffer consisting of Tris-buffered saline (50 mM Tris–HCl, pH 7.5) supplemented with 1% Triton X-100, and then subjected to zymography for 24 h at 37 °C in developing buffer composed of Tris-buffered saline (50 mM Tris–HCl, pH 7.5) supplemented with 5 mM $CaCl_2$ and 1 μM $ZnCl_2$. The gel was then stained with 0.1% Coomassie Brilliant Blue. Gelatinolytic regions were visible as white bands against a blue background. The bands were monochromed and highlighted using WinRoof image analysis software (Mitani Corporation, Fukui, Japan).

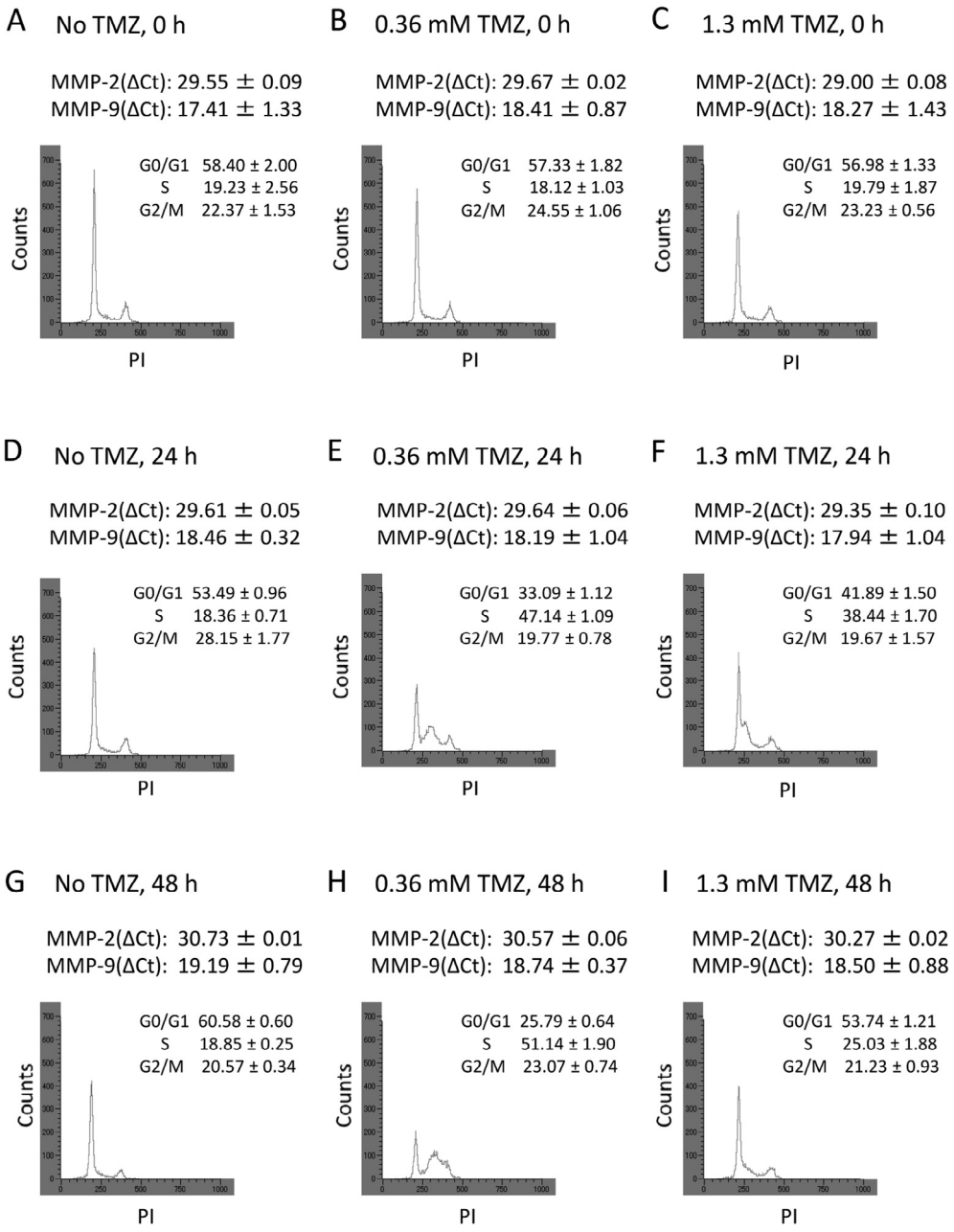

Fig. 2. Effect of TMZ on the cell cycle and on *MMP-2* and *-9* expression in A172 cells. Gene expression was evaluated by semi-quantitative RT-PCR in untreated control cells and in cells exposed to the indicated concentrations of TMZ for the indicated time-periods. Expression levels were normalised to that of *GAPDH* [ΔCt = 35 − (MMP Ct − GAPDH Ct)]. Data represent the mean ± SD of three experiments. G0/G1, G0/G1 phase (%); G2/M, G2/M phase (%); PI, propidium iodide; S, S phase (%).

3. Results

3.1. Cytotoxicity of TMZ

The cytotoxicity assay revealed that exposure to TMZ was cytotoxic to A172 and U373 MG cells (Fig. 1) and facilitated calculation of the IC25 and IC50 values for this compound. These values were 0.36 mM and 1.3 mM for A172 cells, and 0.51 mM and 1.8 mM for U373 MG cells, respectively.

3.2. Effect of TMZ on the cell cycle

As shown in Fig. 2, TMZ treatment altered A172 cell cycle distribution, relative to untreated control cells. Exposure to TMZ (IC25 or IC50) for 24 h increased the fraction of cells in S phase, as compared to control cells. A similar trend was observed for a 48-h treatment. Moreover, the fraction of G1-phase cells was higher following treatment with the TMZ IC50 for 24 or 48 h than it was following exposure to the TMZ IC25. These results indicate that TMZ inhibited the transition of glioma cells to the S and G2/M phases of the cell cycle.

3.3. Effect of TMZ on MMP-2 and -9 transcription

The effect of TMZ on *MMP-2* and *-9* mRNA expression was examined using semi-quantitative RT-PCR in A172 cells. At the time of treatment and 24 h later, *MMP-2* and *-9* mRNA levels remained constant throughout the cell cycle. On the other hand, 48 h after treatment, these mRNA levels were lower in cells exposed to the TMZ IC50 than in those exposed to the IC25 or no TMZ (Fig. 2), although these differences were not statistically significant (Fig. 3).

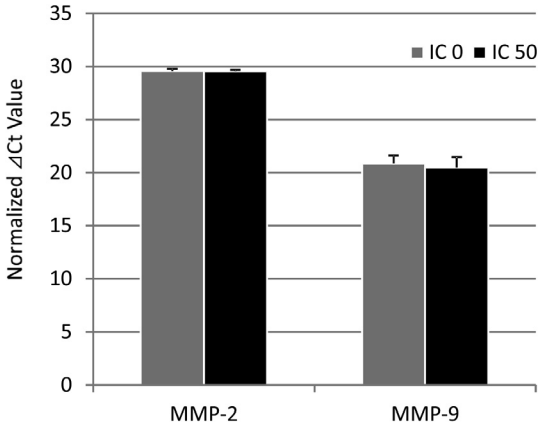

Fig. 3. *MMP-2* and -9 mRNA levels in A172 cells exposed to TMZ. Cells were treated with TMZ (IC50) for 48 h; gene expression was determined by semi-quantitative RT-PCR and normalised to that of *GAPDH* [ΔCt = 35 − (MMP Ct − GAPDH Ct)]. Data represent the mean ± SD of five samples at each time point for each group.

3.4. Activities of MMP-2 and MMP-9

Treatment with TMZ (IC25 or IC50) for 0, 24 and 48 h had no effect on the secretion of either the pro- or cleaved forms of MMP-2 or -9 in A172 (Fig. 4A) or U373 MG (Fig. 4B) cells. Secreted forms of MMP-2 and -9 were detected as bands of 66 and 83 kDa, respectively, indicating that both enzymes were expressed by these glioma cells in their cleaved, activated forms. The secreted pro-forms of MMP-2 also gave rise to a 72-kDa gelatinolytic band, whereas the 96-kDa proMMP-9 was undetectable.

4. Discussion

The present study examined the effect of TMZ on the expression and activities of MMP-2 and -9 in the widely used A172 cells [36] and U373 MG GBM cells. TMZ altered the cell cycle distribution of A172 cells, but did not affect *MMP-2* or *-9* mRNA levels or these enzymatic activities.

Tumour cell invasion is dependent on MMP activation [37,38]. MMP activity, which is regulated by gene transcription, pro-enzyme activation, and inhibition by specific factors [39], can also be activated by other MMPs following secretion into the extracellular space [40,41]. For example, MMP-2 is modulated by MMP-1, -7, -13, -14, -15, -16, -17, -24 and -25, while MMP-9 is modulated by MMP-1, -2, -3, -7, -10 and -13 [31,42,43]. MMP-2 is constitutively expressed in many cell types [44] and is activated by a cell surface mechanism [45] that is largely regulated by pro-enzyme activation [46,47]. On the other hand, the MMP-9 pro-form is secreted into the extracellular space and is activated following proteolytic cleavage by stromelysin and gelatinase A [31,40]. The level

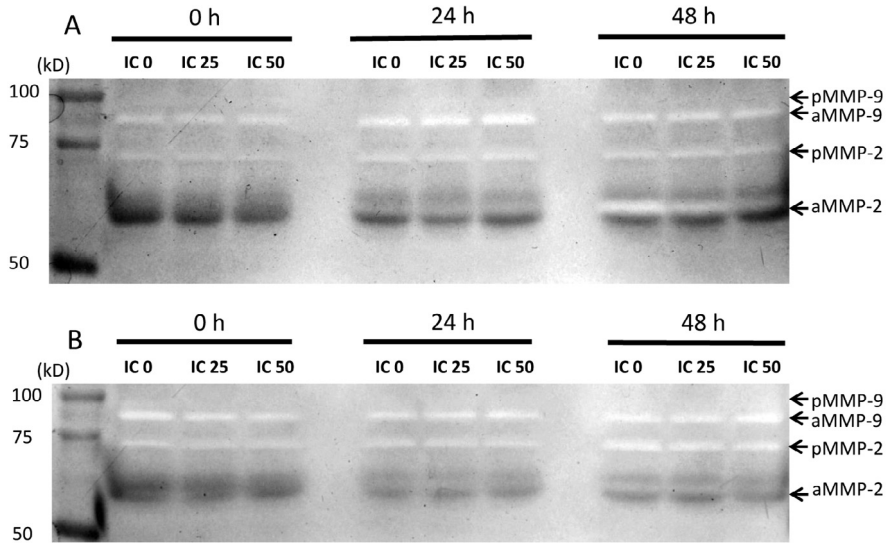

Fig. 4. MMP-2 and -9 enzymatic activities in cells treated with TMZ. The activities of activated MMP-9 (aMMP-9: 83 kDa), proMMP-2 (pMMP-2: 72 kDa) and activated MMP-2 (aMMP-2: 66 kDa) were assessed by gelatin zymography in conditioned culture media from cells exposed to TMZ for 0, 24 or 48 h. ProMMP-9 (pMMP-9: 96 kDa) was undetectable.

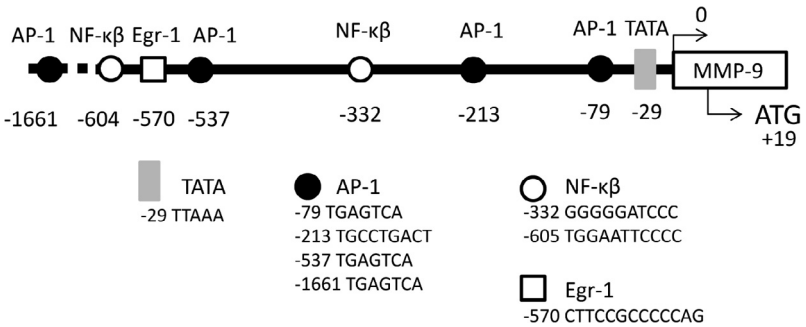

Fig. 5. Schematic depiction of the major regulatory cis-elements in the *MMP-9* gene promoter. The illustration is based on references [31,55]. The 5′ ends of the transcription factor binding sequences (from 5′ to 3′) are indicated as negative numbers.

of *MMP-9* transcription determines MMP-9 activity [33]. Semi-quantitative real-time RT-PCR provides a useful method for evaluating *MMP-2* and *-9* expression, since mRNA levels analysed in this manner were previously shown to correlate with the histological grade of the tumour [48]. Our analysis of *MMP-9* mRNA levels revealed no significant change following exposure to the TMZ IC50 for 48 h, consistent with the lack of effect on MMP-9 enzymatic activity.

Transcriptional factors such as c-fos, c-jun, NF-κβ and egr-1 have putative binding sites on the *MMP-9* promoter (Fig. 5). The activator protein-1 transcriptional complex, composed of c-fos and c-jun, binds to the *MMP-9* promoter and activates *MMP-9* transcription via activation of c-Jun N-terminal kinase [30,31,49,50]. Cytoplasmic NF-κβ rapidly translocates to the nucleus in response to a variety of inflammatory and other stimuli and regulates the transcription of genes associated with inflammation [51]. TMZ was previously shown to increase the nuclear NF-κβ protein level in a manner similar to the effects of ionising radiation on phosphoinositide 3-kinase/Akt/NF-κβ signalling [52]. Egr-1 is a zinc finger transcription factor that regulates genes essential for cell proliferation and differentiation [53,54]. Egr-1, together with NF-κβ, can synergistically activate TNFα-induced *MMP-9* promoter activity [55]. Ionising radiation, which induces DNA damage, rapidly activates egr-1 in order to repair cross-linked DNA [26,56]. Although TMZ activates transcription factors, it did not upregulate MMP-9 activity in the present study.

In clinical trials, an orally administered MMP inhibitor (MMPI) did not improve glioma patient survival following surgery and radiotherapy [57]. However, combined MMPI and TMZ did improve progression-free survival at 6 months in patients with recurrent and progressive GBM [58]. One potential reason for the failure of single MMPI therapy is that these drugs target all MMPs, thereby enhancing tumour vascularisation by suppressing the production of angiogenesis inhibitors, which are generated by MMP activity [59], and stabilising cell-surface MT1-MMP, which paradoxically increases MMP-2 activation [60]. On the other hand, MMPI in combination with TMZ may promote the anti-neoplastic effect of the latter, by suppressing MMP-driven invasiveness. Gliomas release MMP-2 and -9 at the tumour margins; this results in the induction of angiogenesis by upregulation of MMP-9 expression in blood vessels and reconstitution of the basement membrane by MMP-2 expression, leading to tumour cell migration [61]. Combination therapy may create an unfavourable environment for GBM, reducing microvascular proliferation and inducing necrosis.

Our results demonstrate that TMZ does not alter the transcription or activity of MMP-2 or -9. Thus, the anti-neoplastic effect of TMZ is not associated with these two enzymes, and TMZ is not likely to promote tumour invasion in GBM patients. Further study is required to identify drugs that can selectively reduce the activities of MMP-2 and -9; TMZ should be used in combination with these drugs to inhibit tumour invasion and improve the outcome of GBM patients.

Sources of support

This research was supported by The Jikei University Research Fund for Graduate Students. The sponsor had no role in the design or conduct of this research. All of the authors certify that they have no financial or non-financial conflicts of interest with the subject matter or materials discussed in this manuscript.

Acknowledgement

We thank Mayumi Nomura for technical assistance with the experiments.

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