Metronomic treatment of temozolomide inhibits tumor cell growth through reduction of angiogenesis and augmentation of apoptosis in orthotopic models of gliomas

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Abstract. Glioblastoma is a highly angiogenic tumor with a dismal prognosis. Temozolomide (TMZ), a methylating agent is one of the most effective chemotherapeutic agents against glioblastoma. To overcome the problem that most of these tumors become resistant to chemotherapeutic regimens within a year, we investigated the antitumor efficacy of metronomic administration of low-dose TMZ in *in vitro* cell proliferation/ cytotoxicity assay and *in vivo* rat and nude mouse orthotopic glioma model. By *in vitro* assay, we elucidated that C6/LacZ rat glioma cells were more resistant to metronomic treatment of TMZ than U-87MG human glioblastoma cells and bEnd.3 mouse brain endothelial cells. Compared with the conventional chemotherapeutic regimen of TMZ, we found that frequent administration of TMZ at a low dose (metronomic treatment) markedly inhibited angiogenesis as well as tumor growth in a TMZ-resistant C6/LacZ rat glioma model. In addition, metronomic treatment of TMZ significantly augmented apoptosis of tumor cells in this model. For the TMZ-sensitive U-87MG cells, even with a very low dose of TMZ, which is not effective to reduce tumor mass, the metronomic treatment of TMZ reduced the microvessel density, i.e. angiogenesis, in a nude mouse orthotopic model. In conclusion, for both models, the metronomic treatment of TMZ decreased angiogenesis. Especially, in TMZ-resistant glioma cells, this regimen increased apoptosis of tumor cells and decreased tumor growth. The metronomic treatment of TMZ in orthotopic glioma models demonstrated a successful antiangiogenic effect which can overcome the chemoresistance in conventional TMZ chemotherapy.

Introduction

Malignant gliomas are the most common subtypes of rapidly growing primary brain tumors in adults and the most angiogenic human tumors which are characterized by a remarkable proliferative vascular component (1). They have retained their poor prognosis despite aggressive diverse conventional therapeutic approaches, requiring us to find novel therapeutic strategies (2). Particularly, for management of the growth of tumors including gliomas, which are dependent on angiogenesis, i.e. proliferation of microvascular endothelial cells, vascular targeted therapy has been the focus of recent studies.

Temozolomide (TMZ) exhibits broad-spectrum antitumor activity on diverse tumors such as human melanoma, ovarian, colon and brain tumors (3-5). It is a DNA alkylating agent and an imidazotetrazine derivative used in the therapy of malignant gliomas (6,7). Since it has lipophilic property, TMZ is orally available and has shown excellent tissue distribution, including penetration across the blood-brain barrier (3). Although TMZ possesses good antitumor activity, its application in the management of high-grade glioma is limited by various resistant mechanisms (8), which leads many studies to explore the optimization of antitumor efficacy through combination of TMZ with radiation therapy or another agent (6,9-11). It was also reported that the antitumor activity of TMZ is found to be highly schedule-dependent, with multiple administrations being more effective than a single bolus dose (12).

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Recently, many experimental and preclinical studies have suggested that frequent administration *in vivo* of low doses of chemotherapeutic drugs could affect tumor endothelium and inhibit tumor angiogenesis, reducing significant side effects (13-15). These frequent administrations of certain agents with low doses, known as metronomic chemotherapy, especially increase the antiangiogenic activity (16,17).

In this study, we elucidate the antitumor, antiangiogenic and pro-apoptotic activity of metronomic treatment with TMZ through *in vitro* cell proliferation/cytotoxicity assay and *in vivo* orthotopic rat and mouse models of gliomas.

Materials and methods

Cell culture. The rat glioma cell line, C6/LacZ, and bEnd.3 mouse cerebral cortex endothelial cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml), and U-87MG human glioblastoma cells (ATCC) were grown in Eagle's minimal essential medium supplemented with 10% FBS, 2 mM L-glutamine, non-essential amino acids, sodium pyruvate, penicillin (100 units/ml), and streptomycin (100 μg/ml). These cells were maintained at 37˚C in an incubator flushed continuously with 5% CO_2.

In vitro cell proliferation/cytotoxicity assay. Temozolomide (TMZ, temodal) was generously provided by Yuhan Corp. (Seoul, Korea) and dissolved in 10% DMSO. To determine the effectiveness of metronomic TMZ treatment in glioma, we evaluated the effects of various concentrations of TMZ on the proliferation of C6/LacZ and U-87MG cells using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) which is a sensitive non-radioactive colorimetric assay kit for determining the number of viable cells in cell proliferation and cytotoxicity assays. To evaluate the efficacy of metronomic treatment for the glioma associated vascular endothelial cells, we used bEnd.3 which has characteristics of brain endothelial cells (18). After plating these cells into 96-well plates, each TMZ concentration was represented by 5 wells and replicated three times, similar to a previous report which studied the drug effects of prolonged continuous exposures on various cells (15). The cells were treated with TMZ (1-100 μM) for 144 h (1-3x10^3 cells/well in 100 μl of medium). To keep the TMZ concentration constant during the 144-h experiment periods, the medium and the drug were freshly replaced every day.

Animals and orthotopic implantation of tumor cells. For orthotopic glioma model, male Sprague-Dawley (SD) rats (200-250 g) and male Balb/c-nu mice (6 weeks) were housed and maintained under specific pathogen-free conditions in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International and in accordance with the current regulations and standards of the Laboratory Animal Research Center under Samsung Biomedical Research Institute. SD rats and Balb/c-nu mice were anesthetized with intraperitoneal injection of 75 mg/kg ketamin and 5 mg/kg xylazine, and 100 mg/kg ketamin and 10 mg/kg xylazine, respectively. The heads of the anesthetized animals were shaved and disinfected with a solution of 70% ethanol and povidone iodine, after which they were secured in a rodent stereotatic frame. A midline incision was made on the dorsal aspect of the head and the pericranium was laterally to expose the bregma. The screw was implanted into a small drill hole made 3 mm (SD rats) or 2 mm (Balb/c-nu mice) left lateral and 1 mm anterior to the bregma (19). The cells were harvested from subconfluent cultures by brief exposure to 0.25% trypsin and 0.02% EDTA. Only single cell suspensions with 95% viability were used for the *in vivo* implantation. To produce tumors, C6/LacZ and U-87MG tumor cells (1x10^5 cells/10 μl of Hanks balanced salt solution) were injected into the white matter at a depth of 5 mm for SD rats or 2 mm for Balb/c-nu mice, respectively, through a 10-μl Hamilton syringe connected to the manipulating arm of the produced 7-stereotatic device. All injections consisted of a total volume of 10 μl delivered over 12.5 min by a micro-infusion pump.

Drug administration. Before treatment of TMZ, the rats were randomized into four groups (n=10 per group). One experimental group of rats was administered orally with 7 mg/kg TMZ for 5 days (between day 7 and 11 after intracranial implantation). Two groups of rats were respectively administered 1 or 2 mg/kg TMZ via *per os* (p.o.) everyday for 16 days. The control group was treated by injection via p.o. with 10% DMSO. Human U-87MG at nude mice brain is so sensitive to TMZ that we minimized the TMZ dose which starts to show antitumor activity. Then the mice were randomized into five groups (n=15 per group): two conventionally-treated groups were administered orally with 2.5 or 1.25 mg/kg TMZ for 5 days, respectively (between day 14 and 18 after intracranial implantation); two groups were treated orally with each dose of TMZ (0.5 and 0.25 mg/kg) daily for 25 days; and control mice were treated by p.o. with 10% DMSO.

Harvesting of specimens. Seventeen and twenty five days after the inoculation of tumor cells, rats and mice were anesthetized and sacrificed respectively. And then, the brains of these animals were removed and sectioned axially. One section was fixed in 10% buffered formalin and embedded in paraffin, and the other was embedded in OCT compound, frozen rapidly in liquid nitrogen, and stored at -70˚C. The tumor volume was calculated by measuring the section with the largest tumor portion and applying the formula: Length x Width^2 x 0.5 (20).

Immunohistochemistry and quantification of immunostaining. Immunohistochemistry was performed as described previously (20). The microvessel density (MVD) was determined by immunohistological staining using antibody for rat anti-mouse CD31/PECAM-1 (BD Pharmingen, San Diego, CA) in both glioma models. For the quantification of immunostaining for CD31, the number of stained cells was counted in ten random fields at x200 magnification. The apoptotic glioma cells were visualized by a commercially available terminal deoxynucleo-tidyl-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) kit (Intergen Co., Purchase, NY) in C6/LacZ rat glioma orthotopic model. For TUNEL assay, the number of stained cells was counted in ten random fields at x400 magnification.
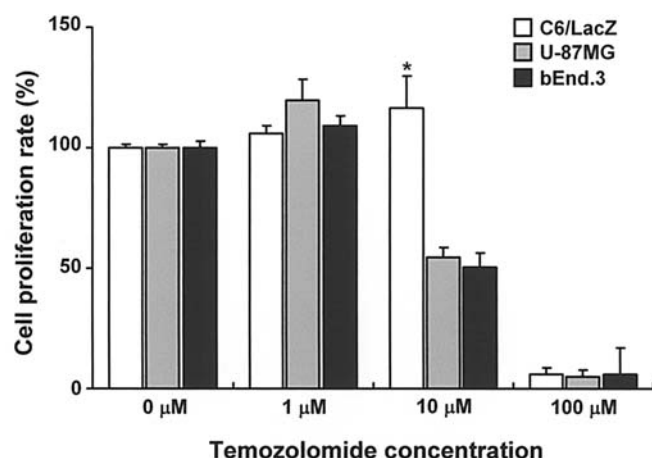
Figure 1. The effects of various doses of TMZ on cell proliferation and cytotoxicity in C6/LacZ, U-87MG and bEnd.3 cells. The proliferation rates of each cell on respective dose of TMZ for 144 h were checked by CCK-8 which is a sensitive colorimetric assay kit for determining the number of viable cells. Each value represents the mean ± SD. *Higher than in U-87MG and bEnd.3 cells treated with 10 μM TMZ (P<0.01).

Data analysis and statistics. Values are presented as means ± SE or ±SD from n animals. Statistical comparisons between groups were performed using Student's t-test or ANOVA. Values of P<0.05 were considered statistically significant.

Results

C6/LacZ cells are more resistant to metronomic treatment with TMZ than U-87MG and bEnd.3 cells on cell proliferation. For determination of the effectiveness of metronomic treatment with TMZ in glioma and glioma associated vascular endothelial cells, we evaluated the effects of various concentrations of TMZ on cell proliferation of two glioma cells and bEnd.3 cells using CCK-8 (Dojindo Laboratories). Significant inhibition of cell proliferation was observed in C6/LacZ, U-87MG and bEnd.3 cells treated with 100 μM of TMZ for 144 h, respectively (Fig. 1). In contrast, the proliferation rate of C6/LacZ cells (P<0.01) was not changed in the treatment

group of 10 μM TMZ compared with those of other groups of cells (Fig. 1). In detail, the value of IC_{50} for C6/LacZ cells (104.14) was shown to approximately 10.6-fold augmentation compared with those for U-87MG (9.85) and bEnd.3 (9.72) cells, which suggested that C6/LacZ cells were more resistant to TMZ than U-87MG and bEnd.3 cells. According to the *in vitro* data, C6 glioma cells of rat brain seem to be resistant to TMZ chemotherapy, whereas tumor associated proliferating endothelial cells seem to be sensitive to antiangiogenic TMZ chemotherapy.

Metronomic treatment with TMZ inhibits tumor growth in C6/LacZ rat glioma orthotopic model. To find the optimal dose effect of TMZ in an animal model, we orally administered the same dose of TMZ (7 mg/kg) or 10% DMSO (control) for 5 days to rats and mice after intracranial implantation of C6/LacZ and U-87MG cells respectively.

Compared with control (median volume, 74.4 mm³), the tumor volume in rats treated with conventional TMZ chemotherapy was slightly reduced (median volume, 52.4 mm³) on the 16th day after the inoculation of tumor cells (Table I), while tumor formation in mice was completely blocked on the 25th day after the inoculation of tumor cells (data not shown), which means that tumor was not grown in 7 mg/kg TMZ-treated mice. Therefore, C6 glioma in rat brain was relatively resistant to the TMZ chemotherapy in accordance with the results of the cell proliferation experiment (Fig. 1).

In both a rat and mouse animal model, there were no signs of toxicity of drug administration, such as body weight loss, in either group (data not shown).

To determine the antitumor activity of TMZ on gliomas, we measured the tumor volume after each treatment of TMZ in a C6/LacZ rat glioma orthotopic model (Table I, Fig. 2A). Control rats had the largest brain tumors (median volume, 74.4 mm³). In contrast, all groups treated with TMZ had reduced brain tumor volumes. The tumor masses of rats administered 7 mg/kg TMZ for 5 days were reduced by 30% compared to those of controls (median volume, 52.4 mm³), whereas the tumor masses of rats which were administered daily with 2 mg/kg TMZ were markedly reduced by 70%

Table I. Tumor volume, microvessel density (MVD) and apoptosis index in a C6/LacZ rat glioma model.

Treatment group	Tumor volume (mm³)		MVD (no.)		TUNEL (no.)	
	Median	Range	Median	Range	Median	Range
Control	74.4	1.5-292.9	23	16-35	2	1-3
7 mg/kg TMZ for 5 days	52.4	19.6-255.6	19	10-30	5[c]	2-13
2 mg/kg TMZ daily	22.3[a]	1.3-79.5	12[b]	7-24	14[d]	6-23
1 mg/kg TMZ daily	49.7	0.0-186.5	22	15-33	2	0-5

C6/LacZ rat glioma cells (1x10⁵) were injected into the male Sprague-Dawley (SD) rat brain. The respective groups of rats were treated by p.o. injection with 7 mg/kg temozolomide (TMZ) for 5 days or oral administration with 1 or 2 mg/kg TMZ daily. Control group of rats was administered orally with 10% DMSO. No. is the number of positive staining cells. [a]Smaller than in gliomas of control group animals (P<0.05). [b]Lower than in gliomas of control animals (P<0.001). [c]Higher than in gliomas of control group animals (P<0.001). [d]Higher than in gliomas of the other groups of animals (P<0.05).
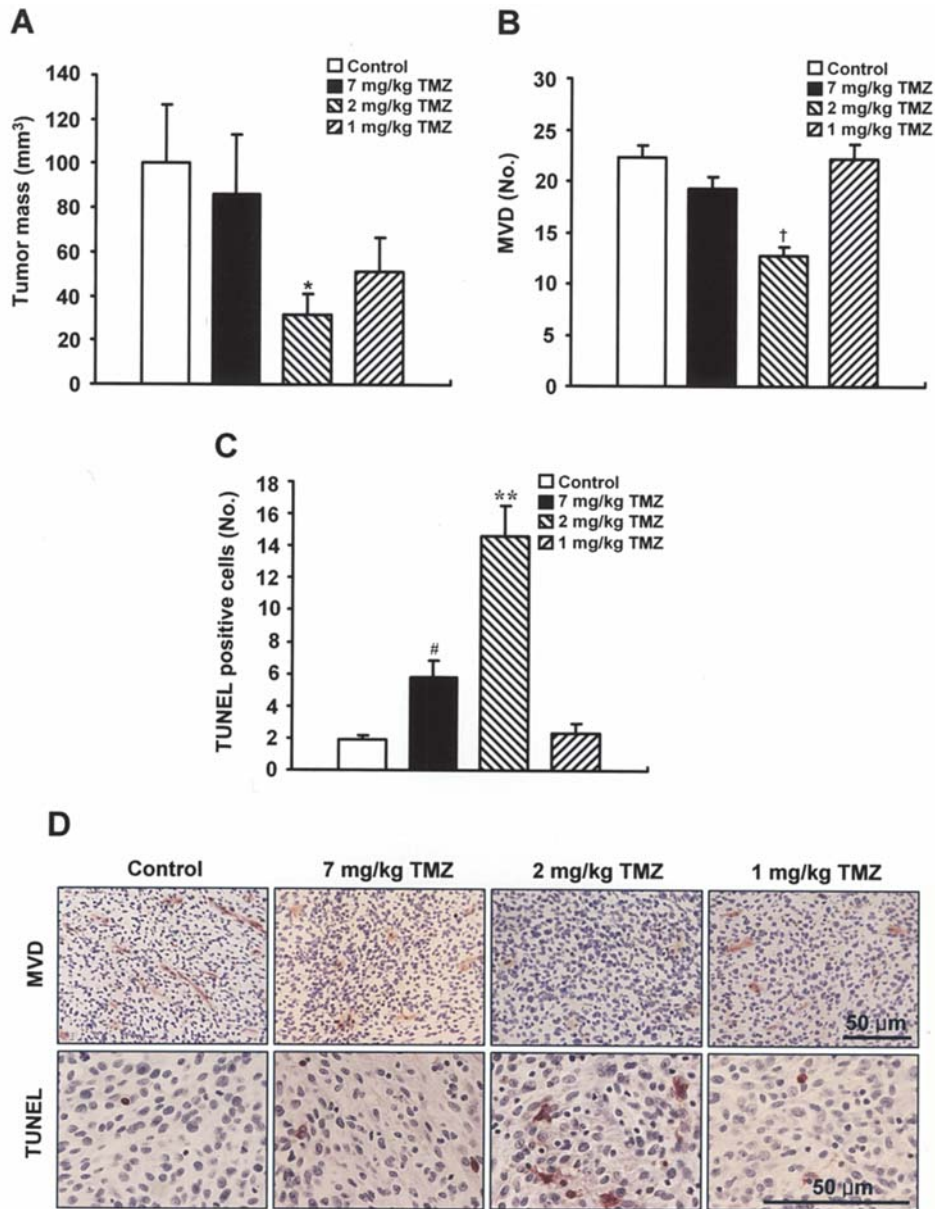
Figure 2. The effects of TMZ on C6/LacZ rat gliomas in orthotopic model. Tumor volume (A), microvessel density (MVD, B) and TUNEL positive cells (C) are graphed respectively. Each value represents the mean ± SE (n=10). The values of mean in this figure are different from those of the median in Table I. *Smaller than in gliomas of control group animals (P<0.05). †Lower than in gliomas of control (P<0.001). #Higher than in gliomas of control (P<0.001). **Higher than in gliomas of the other group animals (P<0.05). (D) Paraffin sections of the tumors were stained for endothelial cells using anti-CD31 antibody (upper pannels) and apoptotic cells using TUNEL assay (lower pannels), respectively.

compared with those of controls (median volume, 22.3 mm³, P<0.05). Furthermore, the reduction of tumor mass was also observed, even with half the amount of the total dose used for conventional TMZ chemotherapy (median volume, 49.7 mm³).

Metronomic treatment with TMZ inhibited angiogenesis and induced apoptosis in an orthotopic rat glioma model. The microvessel density (MVD) and apoptotic cells in a C6/LacZ rat glioma orthotopic model were determined by immuno-histological staining with CD31 antibody and TUNEL kit, respectively.

The number of blood vessels stained with CD31 was decreased in all groups treated with TMZ compared with those of controls (Table I, Fig. 2B and D). The group of rats daily administered with 2 mg/kg TMZ showed 48%

decrease (P<0.001) in MVD relative to control, which is significantly different compared with the other groups. These results demonstrated that the protracted treatment of TMZ with proper low dose was more effective on inhibition of tumor growth and angiogenesis than the relatively high dose of conventional consecutive TMZ chemotherapy for 5 days. C6 glioma in SD rats, which is TMZ chemoresistant in conventional chemotherapy, became sensitive to a frequent low-dose schedule.

The apoptotic cells determined by TUNEL assay were observed in rats treated with each dose of TMZ. While TUNEL-positive cells were rarely detected in control, TUNEL-stained cells were shown in all groups treated with TMZ (Table I, Fig. 2C and D). Moreover, in the group of rats metronomically administered 2 mg/kg TMZ, the number of

Table II. Tumor volume and MVD in human U-87MG glioma xenograft model.

Treatment group	Tumor volume (mm^3)		MVD (no.)	
	Median	Range	Median	Range
Control	178.0	4.5-180.0	11	6-16
2.5 mg/kg TMZ for 5 days	21.6[a]	0.8-136.8	11.5	6-16
1.25 mg/kg TMZ for 5 days	80.1	0.0-123.7	12.5	8-22
0.5 mg/kg TMZ daily	18.5	2.5-126.3	7[b-d]	1-14
0.25 mg/kg TMZ daily	105.8	84.5-171.7	8[b,d]	2-15

U-87MG human glioma cells (1x10^5) were injected into the male Balb/c-nu mice brain. Two groups of mice were treated orally with 2.5 or 1.25 mg/kg TMZ for 5 days. The other two groups of mice were administered orally with 0.5 or 0.25 mg/kg TMZ for everyday. The control group of mice was treated by p.o. injection with 10% DMSO. No. is the number of positive staining cells. [a]Smaller than in gliomas of control group animals (P<0.05). [b]Lower than in gliomas of control group animals (P<0.05). [c]Lower than in gliomas of mouse group administered 2.5 mg/kg TMZ (P<0.05). [d]Lower than in gliomas of group animals treated with 1.25 mg/kg TMZ (P<0.001).

apoptotic cells showed a 7-fold increase (P<0.05) compared with that in controls (Table I, Fig. 2C and D).

Metronomic treatment with TMZ inhibits angiogenesis in U-87MG human glioblastoma orthotopic xenograft model. We also examined the effect of TMZ on the tumor volume and MVD in a U-87MG human glioblastoma orthotopic xenograft model according to treatments for respective doses of TMZ (Table II, Fig. 3). Since U-87MG in the nude mouse brain is sensitive to conventional TMZ chemotherapy, we reduced and treated it conventionally with TMZ at one third of the dose having an optimal effect and with TMZ divided into an everyday schedule at the same dose as conventional chemotherapy. Although there is no significant difference between the conventional and metronomic dose in tumor volume in this model, the antiangiogenic effect is more prominent in the metronomic dose schedule (Table II, Fig. 3A and B). In detail, the number of vessels stained with CD31 in mouse groups treated with TMZ for 5 days showed the similarity compared with that of controls, while the number of CD31 staining blood vessels in mouse groups metronomically treated with TMZ for everyday were decreased by 64% (0.5 mg/kg TMZ daily, P<0.05, calculated by each value of median) and 73% (0.25 mg/kg TMZ daily, P<0.05) compared with those of controls (Table II, Fig. 3B and C).

Discussion

In this study, the effect of metronomic treatment with TMZ, one of the most commonly used chemotherapy agents for
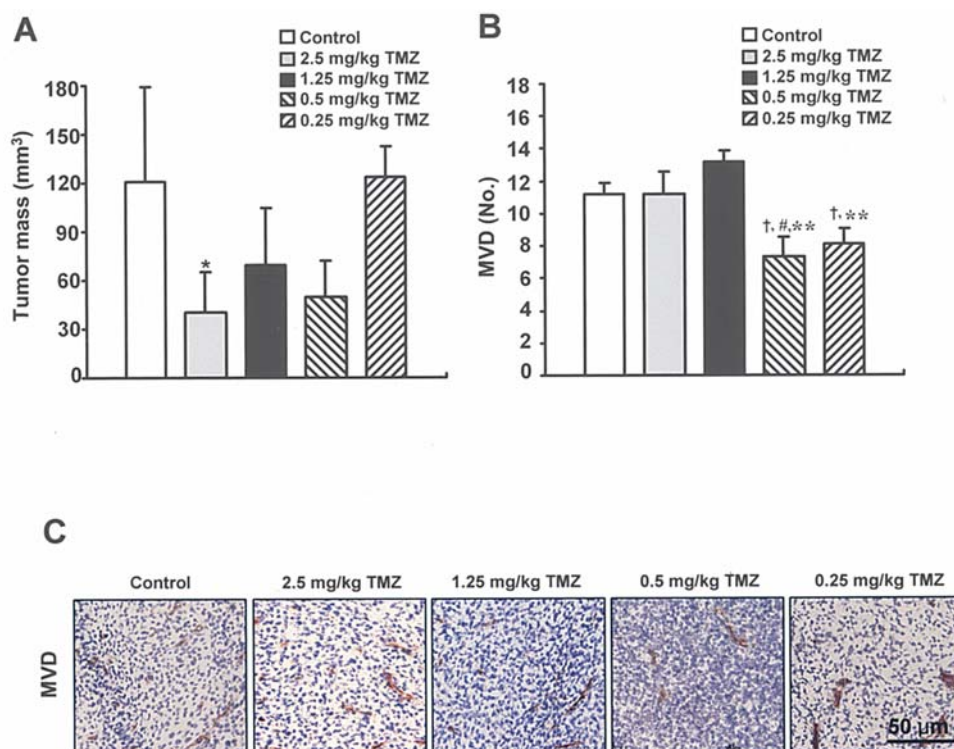

Figure 3. The effects of TMZ on a U-87MG human glioma xenograft model. Tumor volume (A) and MVD (B) are graphed respectively. Each value represents the mean ± SE (n=15). The values of mean in this figure are different from those of median in Table II. *Smaller than in gliomas of control group animals (P<0.05). †Lower than in gliomas of control (P<0.05). #Lower than in gliomas of mouse group administered 2.5 mg/kg TMZ (P<0.05). **Lower than in gliomas of group animals treated with 1.25 mg/kg TMZ (P<0.001). (C) Paraffin sections of the tumors were stained for endothelial cells using anti-CD31 antibody.

malignant gliomas (6,7), is investigated by cell proliferation assay *in vitro* and with rat and mouse orthotopic glioma model *in vivo*. Previous studies reported that antiangiogenic effects of different doses of TMZ are determined by the *in vivo* chorio-allantoic membrane (CAM) assay, and HUVEC-based *in vitro* Matrigel, adhesion and proliferation assays (21). It was also demonstrated that TMZ inhibits tumor growth by inducing apoptosis in a three dimensional cell culture model of the glioma cell lines (22). In this report, we originally executed the metronomic chemotherapy of TMZ and optimized the antitumor activity of TMZ in orthotopic glioma models. We elucidate that frequent administration of TMZ at a low dose significantly inhibits the growth of tumor in relatively TMZ-resistant rat glioma. The mechanism of this antitumor activity is accompanied by the reduction of angiogenesis and the induction of apoptosis in TMZ-resistant and -sensitive animal models.

Clinically, concomitant radiation and TMZ chemotherapy shows a survival gain in glioblastoma patients. One of the reasons is that an everyday low-dose treatment schedule during radiation therapy for 6 weeks has a potent antiangio-genic or antivascular effect on glioblastoma, which has the most prominent endothelial proliferation compared with other cancers (23,24).

Recently it was suggested that the endothelial cells in the vascular bed of tumor are more susceptible to chemothera-peutic agents than resting endothelium, because they have significantly higher proliferation rates than the normal endo-thelium in the rest of the body (25). In addition, before reaching tumor cells, these agents must meet microvascular endothelial cells in the tumor beds. Furthermore, endothelial cells are genetically stable, so they should be more susceptible to the apoptotic effects of chemotherapeutic agents than tumor cells (25). Therefore, the apoptotic effects of cytotoxic chemotherapy on proliferating vascular endothelial cells could contribute to the antitumor efficacy of chemotherapy.

Metronomic chemotherapy, continuous chemotherapeutic agent administration on a low-dose antiangiogenic schedule, exposes drugs to endothelial cells in the tumor beds and can induce apoptosis of endothelial cells preceding that of tumor cells. Various kinds of cytotoxic agents, according to the theory mentioned above, can be applied to diminish or to regress the tumor mass in experimental models and some patients (25-28). Similarly we hypothesized that the con-ventional chemotherapy of TMZ required a treatment-free interval to allow the recovery of tumor cell growth, implying that the presence of microvascular endothelial cells in the tumor bed involves resuming their proliferation and supporting tumor regrowth. Glioma-bearing animals conventionally treated with TMZ would have large tumors with a diminished response to TMZ which could acquire drug resistance. In contrast, when TMZ was administered more frequently and at lower doses without a prolonged treatment-free interval, tumors regressed without other toxicity in animals.

In conclusion, we demonstrate that frequent and continuous treatment with a low dose of TMZ showed high antitumor effects through, at least in part, its angiogenic and pro-apoptotic activity. Therefore, it should be strongly stressed that low-dose TMZ treatment frequently becomes a potential candidate as chemotherapy for malignant gliomas.

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