Metronomic treatment of temozolomide increases anti-angiogenicity accompanied by down-regulated O^6-methylguanine-DNA methyltransferase expression in endothelial cells

KYUNG-KON KO[1], EUN-SANG LEE[1], YOUNG AE JOE[2] and YONG-KIL HONG[1,2]

[1]Department of Neurosurgery, and [2]Cancer Research Institute, Seoul St. Mary's Hospital, The Catholic University of Korea College of Medicine, Seoul, Republic of Korea

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Abstract. Metronomic chemotherapy is a continuous low-dose administration of chemotherapeutic agents to minimize toxicity and target tumor-associated endothelial cells. This therapy is beneficial to anti-angiogenic efficacy which is linked to the inhibition of tumor growth. In the present study, we compared the anti-angiogenicity of temozolomide in human umbilical vein endothelial cells (HUVECs) between conventional and metronomic treatment. Metronomic treatment of temozolomide (TMZ) (6.25 and 12.5 μM) showed increased inhibition of the proliferation of HUVECs compared to an equivalent conventional treatment of TMZ. The differential effects between conventional and metronomic treatment of TMZ were also noted in cell migration and angiogenic tube formation. Notably, the expression level of O^6-methylguanine-DNA methyltransferase (MGMT) was markedly reduced in the HUVECs treated with metronomic TMZ (12.5 and 25 μM) compared to cells treated with conventional treatment of TMZ. Accordingly, HUVECs treated with metronomic treatment of TMZ were more sensitive to TMZ treatment. Taken together, metronomic chemotherapy with TMZ enhances the inhibition of angiogenesis accompanied by the down-regulation of MGMT expression in endothelial cells when compared to conventional chemotherapy.

Introduction

Temozolomide (TMZ) is an oral imidazotetrazine methylating agent that undergoes spontaneous chemical conversion to 3-methyl-(triazen-1yl)-imidazole-4-carboxamide (MTIC) at physiological pH (1). A number of studies have reported that methylation of the O^6-lesion of guanine through TMZ leads to various cellular responses, including cytotoxicity and anti-angiogenesis, which is due to the generation of DNA single-strand gaps and/or double-strand breaks during DNA synthesis and results from the inhibition of angiogenesis-associated genes, respectively (2-4). Since TMZ readily crosses the blood-brain barrier, it is the favorable chemotherapeutic drug for glioma patients (5). Preclinical and clinical investigations have demonstrated that the anticancer activity of TMZ depends on the administration schedule and dose (6,7). Although the anticancer activity of TMZ has been established at a maximum tolerated dose (MTD), the effect was optimized by metronomic administration at low doses and frequent schedule for the purpose of avoiding of drug resistance (8). Metronomic treatment of TMZ was found to lead to an anti-angiogenic effect accompanied by a relatively lower density of microvessels in orthotopic glioma models (9). One metronomic chemotherapy study suggested that this scheduling overcomes chemoresistance in patients with recurrent TMZ-refractory glioblastoma (GBM) without any toxicity (10). In addition, it was found that metronomic TMZ scheduling was capable of inhibiting angiogenesis in *in vivo* and *in vitro* studies using endothelial cells (11).

O^6-methylguanine-DNA methyltransferase (MGMT) is a DNA-repair protein that plays a pivotal role in cellular resistance to alkylating agents, as MGMT removes methyl and alkyl groups at the O^6-position of guanine which lead to DNA damage through covalent linking with an internal cysteine amino acid present within the MGMT protein (12). In fact, it has been well documented that MGMT activity determines the chemotherapeutic efficacy in GBM. MGMT prevents apoptosis originated from O^6-methyl-guanine (O^6-MeG) through TMZ treatment in glioma cells (4). In resistant glioma cells, IFN-β down-regulates MGMT expression, which results in sensitization to TMZ (13). Moreover, clinical research has reported that methylation of the MGMT promoter is useful to predict the responsiveness to alkylating agents (14). Recently, significant inhibition of glioma cell proliferation and survival was

Correspondence to: Dr Yong-Kil Hong, Department of Neurosurgery, Seoul St. Mary's Hospital, The Catholic University of Korea, Banpo-dong 505, Seocho-gu, Seoul 137-701, Republic of Korea
E-mail: hongyk@catholic.ac.kr

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found to be dependent on the MGMT status (15). Notably, one clinical study found that significant and prolonged depletion of O^6-alkylguanine-DNA-alkyltransferase (AGAT) activity was produced by metronomic chemotherapy of TMZ (16). We thus speculated that our metronomic schedule would regulate MGMT expression.

In the present study, we found that down-regulated MGMT expression resulted from metronomic treatment of TMZ in human umbilical vein endothelial cells (HUVECs). We also demonstrated that metronomic treatment of TMZ induced an anti-proliferative effect by down-regulating MGMT expression. Furthermore, reduced cell migration and tube formation were observed in HUVECs cultured with metronomic treatment with TMZ compared to conventional treatment with TMZ. Therefore, we suggest that metronomic chemotherapy with TMZ benefits the inhibition of angiogenic activity compared to conventional chemotherapy, and down-regulates MGMT expression, which may be associated with anti-angiogenicity in endothelial cells.

Materials and methods

Isolation of human umbilical vein endothelial cells and culture condition. HUVECs were isolated from human cords according to a previous study (17). IRB-approved cells were cultured in specific endothelial cell growth media (M199) supplemented with 20% fetal bovine serum (FBS; Gibco, USA), 30 μg/ml endothelial cell growth supplement (Sigma, St. Louis, MO, USA), 90 μg/ml heparin and 1% antibiotics. HUVECs were maintained at 37˚C in an atmosphere with 5% CO_2. HUVECs for all of the experiments were passaged four to six times.

Drug preparation and determination of the in vitro IC_{50} value of TMZ. TMZ was supplied from Schering-Plough (Kenilworth, NJ, USA). It was dissolved in 0.5% DMSO to produce a 20 mg/ml stock solution. To determine the *in vitro* IC_{50} value of TMZ for HUVECs, the cells were treated with 50, 100, 200, 400 and 800 μM of TMZ. The IC_{50} value (372.2 μM) was calculated using the following formula: (X_2-X_1) x $(50-Y_1)/(Y_1+Y_2)$ + X_1; where X_1 and X_2 indicate the high and low dose, respectively. '50' indicates a global cell growth of 50%, and both Y_1 and Y_2 indicate the mean percentage of X_1 and X_2, respectively (27).

HUVEC proliferation assay. HUVEC proliferation assay was performed using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan), which is a sensitive non-radioactive colorimetric assay kit. HUVECs were seeded at $1x10^3$ cells in 96-well plates and incubated at 37˚C, overnight. After 1 day, HUVECs were treated with 37.5, 75, 150 and 300 μM of TMZ conventionally and 6.25, 12.5, 25 and 50 μM of TMZ metronomically, which was carried out using one-time and six-time treatments, respectively. After TMZ treatment, the cells were cultured continuously for 144 h. The optical density was measured at 450 nm using an ELISA reader (Molecular Devices, San Francisco, CA, USA). Another proliferation assay was performed using HUVECs which were cultured with non-treatment, conventional treatment of TMZ and metronomic treatment of TMZ for 144 h. The cells were cultured by the method mentioned above. Briefly, the cells were harvested at 144 h and then cultured in 96-well plates for 1 day at 37˚C (5% CO_2, humidity). The cells were treated with 100 μM of TMZ. The anti-proliferative effect was determined in the HUVECs after an additional culture for 72 h.

Migration assay. A migration assay was performed using a modified Boyden chamber (Neuro Probe, Inc., Cabin John, MD, USA) according to the manufacturer's recommendation. The underside of the polycarbonate membrane (8 μm) was coated with 0.1% gelatin overnight at room temperature (RT). The membrane was placed over the lower chambers containing 0.1% BSA, 90 μg/ml heparin supplemented with 2 ng/ml VEGF ($VEGF_{165}$; R&D Systems, Wiesbaden, Germany) in EBM2, and the upper chamber was then assembled. After assembling, HUVECs treated with TMZ by a conventional or metronomic schedule were incubated in free EBM2 for 4 h. The cells ($2x10^4$) were transferred to the upper chamber and cultured for 5 h at 37˚C with 5% CO_2 to induce cell migration. Fixation and staining with Diff-Quick solution (Sysmex, Kobe, Japan) were performed. Measurement of the migrated cells was also performed by manual counting after photography.

Tube formation assay. Unpolymerized Matrigel (150 μl; BD Bioscience) was placed in pre-chilled 48-well plates and incubated for 30 min at 37˚C. HUVECs ($3x10^4$) treated with TMZ by the conventional or metronomic schedule were transferred onto the solidified Matrigel. After incubation for 18 h at 37˚C in 5% CO_2, tube formation was observed, and images of five representative fields were captured. Tube lengths were determined using Image J (http://rsb.info.nih.gov/ij/).

Western blot analysis. Protein was extracted from the HUVECs treated with the conventional or metronomic TMZ schedule. Electrophoresis was performed on 10% polyacrylamide gels, and the proteins were transferred onto nitrocellulose membranes (Pall Co., Pensacola, FL, USA) for 90 min at 4˚C. The membrane was blocked with blocking solution (5% skim milk; BD Bioscience), 0.1% Tween-20, 1X TBS for 1 h at RT. The membrane was incubated with anti-MGMT (1:2,000, mouse; Chemicon International Inc.) and anti-β-actin (1:10,000, mouse; Sigma-Aldrich) antibodies overnight at 4˚C. The membrane was washed three times and then incubated with a secondary antibody (1:10,000, goat anti-mouse IgG-conjugated HRP; Cell Signaling, USA) for 2 h at RT. Blots were developed by enzyme-linked chemiluminescence (ECL detection kit; Amersham Biosciences).

Results

Metronomic treatment of TMZ improved anti-angiogenic efficacy in HUVECs. A distinction between conventional and metronomic chemotherapeutic efficacy of TMZ against angiogenicity was assessed by proliferation, cell migration and angiogenic tube formation in HUVECs. First, we evaluated the proliferative effect between conventional and metronomic treatment of TMZ using CCK-8 (Dojindo Laboratory). Prior to the evaluation of the proliferative effect, we determined the *in vitro* IC_{50} value of HUVECs for growth inhibition. HUVECs were cultured for 120 h after treatment with 0-800 μM TMZ, and the IC_{50} value was determined to be 372.2 μM. As shown

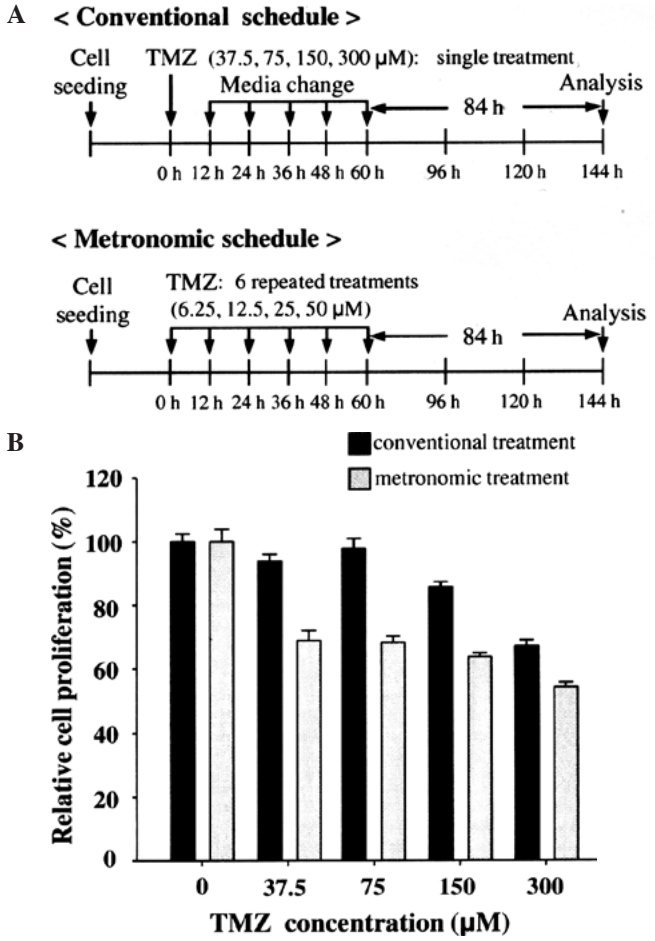

Figure 1. HUVEC proliferation was decreased by metronomic treatment of TMZ. (A) Schematic schedule of temozolomide treatments. HUVECs were treated with TMZ conventionally and metronomically after being seeded. The cells were repeatedly treated with culture medium containing TMZ six times every 12 h for metronomic treatment and subjected to single treatment and refreshment with culture medium every 12 h for conventional treatment. After TMZ treatments for 3 days, the cells were continuously cultured with fresh medium for 3 days. All experiments, including cell migration, tube formation and proliferation, were performed for 144 h. (B) Comparison of the anti-proliferative effect between conventional and metronomic treatment of TMZ in HUVECs. HUVECs treated with 12.5 or 25 μM of TMZ metronomically showed significant inhibition of proliferation compared to an equivalent conventional treatment of TMZ. Each value represents the mean ± SD.

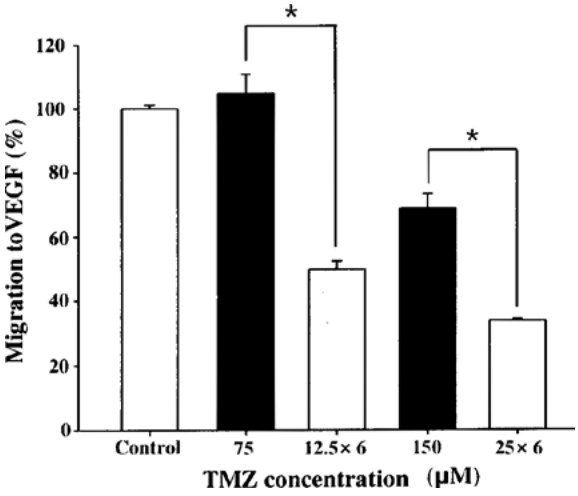

Figure. 2. Increased inhibition of cell migration of HUVECs upon metronomic treatment of TMZ. HUVECs were plated into the upper chamber to induce cell migration towards the lower chamber containing $VEGF_{165}$ supplemented with EBM2 medium. Cell migration was reduced in the HUVECs cultured with metronomic treatment of TMZ compared to conventional treatment of TMZ. *P<0.01.

in Fig. 1B, metronomic treatment of TMZ significantly inhibited cell proliferation. The cell numbers of HUVECs metronomically treatment with 12.5 and 25 μM TMZ after 144 h were ~6.4- and 2.4-fold less than those treated with the conventional TMZ treatment, respectively. Intriguingly, the anti-proliferative effects of a metronomic treatment of 6.25 μM TMZ and a conventional treatment of 300 μM TMZ were almost identical. Therefore, HUVEC proliferation was markedly decreased by the metronomic treatment of TMZ compared to the conventional treatment of TMZ, suggesting that the side effects of conventional chemotherapy of TMZ may be avoided.

To measure the anti-angiogenic activity of the metronomic treatment of TMZ, cell migration assay was performed using a Boyden chamber with slight modification. The results showed that the numbers of cells which migrated towards the VEGF-coated bottom were significantly reduced, ~41 and 67% at 12.5 and 25 μM TMZ, respectively, compared to the HUVECs which were treated with the conventional TMZ schedule (Fig. 2).

An additional anti-angiogenic activity was assessed through a tube formation assay using a Matrigel basement membrane matrix (BD Bioscience). HUVECs treated with the metronomic and conventional TMZ protocols for 144 h were then cultured on a Matrigel matrix to induce alignment and formation of hollow tube-like structures. Upon metronomic treatment with 25 μM TMZ, tube formation of HUVECs was markedly inhibited compared with the tubal formation of HUVECS exposed to the conventional treatment with 150 μM TMZ, indicating that conventional treatment of TMZ does not inhibit tube formation of tumor-associated endothelial cells (Fig. 3). Taken together, metronomic chemotherapy of TMZ is beneficial for anti-angiogenicity by inhibiting cell proliferation, cell migration in VEGF and angiogenic tube formation.

Metronomic treatment with TMZ inhibits MGMT expression in HUVECs. Tolcher *et al* reported that AGAT activity is decreased by prolonged administration of TMZ in peripheral blood mononuclear cells (PBMCs) (16). Since the action of therapeutic methylating agents, including TMZ, dacarbazine, streptozotocin and procarbazine, against MGMT activity has been investigated in various cell types and disease models (18-20), we speculated that MGMT may be involved in the proliferation of HUVECs. We therefore examined the expression level of MGMT through both conventional and metronomic treatment of TMZ using immunoblot analysis using cells harvested at 144 h. The results showed that the expression level of MGMT was significantly reduced by metronomic treatment with TMZ, particularly at 25 μM TMZ, although MGMT expression upon conventional treatment of TMZ was nearly similar (Fig. 4A). Furthermore, we assessed an additional metronomic therapeutic efficacy of TMZ in HUVEC proliferation which may be related to MGMT expression level. As a result of treatment with 100 μM TMZ,

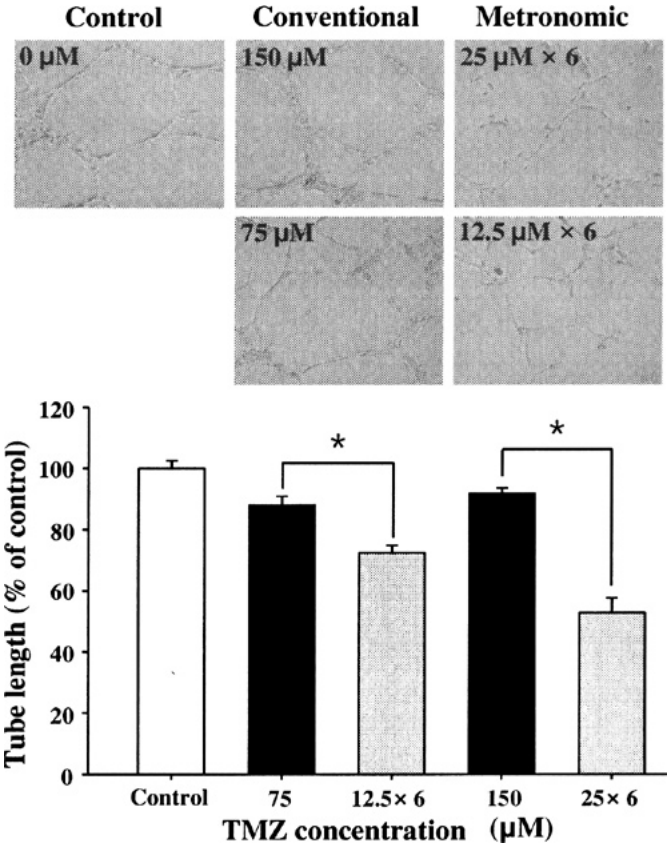

Figure 3. Increased inhibition of tube formation in HUVECs upon metronomic treatment of TMZ. Tube length was measured in five representative fields in images obtained from photographs. Inhibition of tube formation was marked in HUVECs that were treated with metronomic treatment of TMZ compared to conventional treatment of TMZ. *P<0.01.

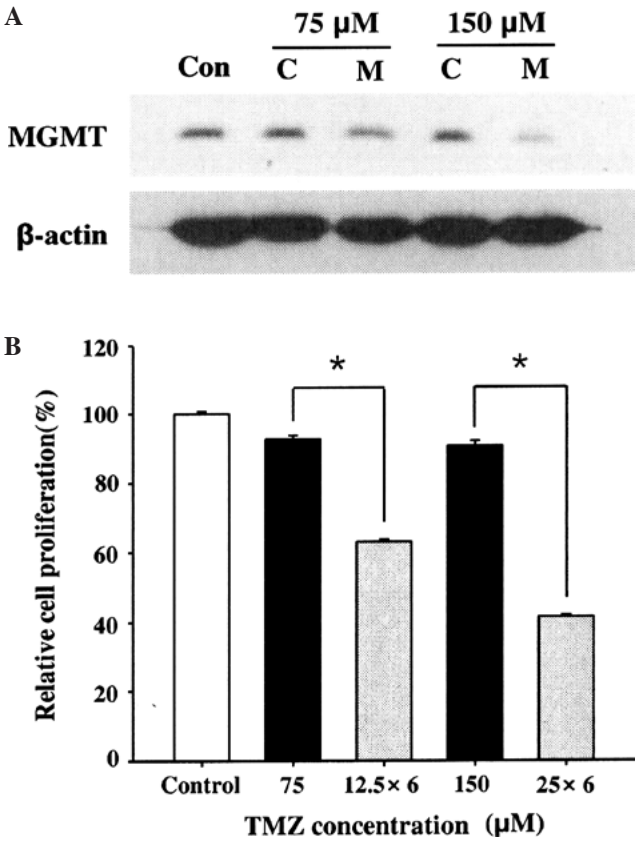

Figure 4. Down-regulation of MGMT expression upon metronomic treatment of TMZ. HUVECs were cultured with conventional or metronomic treatment of TMZ for 144 h. (A) After harvesting the cells, half of them were subjected to Western blot analysis. Immunoblot analysis showed that treatment of metronomic doses, 12.5 and 25 μM of TMZ, down-regulated MGMT expression in HUVECs. C, conventional treatment; M, metronomic treatment. (B) The other half was plated onto a new dish, cultured for 1 day and then treated with TMZ (100 μM) for 72 h. The rate of cell growth was lower in the HUVECs cultured with metronomic treatment of TMZ compared to conventional treatment of TMZ. *P<0.01.

HUVECs that were treated with a metronomic TMZ schedule were consistently inhibited in cellular growth compared to HUVECs that were cultured with the conventional TMZ schedule (Fig. 4B). Thus, we identified that metronomic treatment of TMZ deregulates MGMT expression in HUVECs, and MGMT inactivity may lead to a decrease in proliferation of endothelial cells.

Discussion

Conventional chemotherapy of anticancer drugs has anti-tumor efficacy accompanied by anti-angiogenicity (8). Despite that conventional chemotherapy promises a successive and curative effect, it also gives rise to side effects, such as cytotoxicity and tolerance resulting in disease recurrence. By contrast, metronomic treatment has become a suitable chemotherapeutic method which includes the advantages of both the possibility of combination treatment and the prevention of drug resistance. As metronomic chemotherapy was shown to exhibit anti-angiogenic potential, including apoptosis or inhibition of proliferation in endothelial cells, our aim focused on the mechanism of metronomic TMZ chemotherapeutic efficacy by investigating anti-angiogenicity and evaluating the MGMT expression level.

Inhibition of tumor angiogenesis has become a popular anti-tumor target through the use of chemotherapeutic drugs, such as TMZ, methotrexate (21), paclitaxel (22), vinblastine (23), bevacizumab (24) and doxorubicin (25), either directly or indirectly by the inhibition of cell proliferation, migration and blood vessel formation. Studies on the metronomic chemotherapy of anti-angiogenic drugs have shown that paclitaxel inhibits the proliferation of HUVECs at low doses, although doxorubicin inhibits angiogenesis at low doses without inhibition of cell migration and tube formation (22,26). Since each drug has different therapeutic effects on endothelial cells for tumor angiogenesis, metronomic treatment used in combination with other drugs is possible to improve the therapeutic effect. Regarding combination treatment, an anti-tumor effect was found to be markedly increased by a combination of both TMZ and bevacizumab in a human GBM orthotopic xenograft model (27), indicating that metronomic treatment of TMZ may have the advantage of a synergistic improvement in the therapeutic effect by co-administration with other treatments.

We assumed that the metronomic chemotherapeutic effect appears to be dependent on the IC_{50} value according to the following. Kurzen *et al* reported the IC_{50} of TMZ to be approximately 250 μM; inhibition of HUVEC proliferation occurs at a minimum dose of 25 μM of TMZ, and low doses

in a range of 2.5-10 μM do not significantly inhibit HUVEC proliferation (11). By contrast, Lam *et al* (28) reported that a significant anti-proliferative effect in HUVECs was observed upon treatment of 2.5 μM TMZ treatment with an IC_{50} value of 6.6 μM. In the present study, we determined the *in vitro* IC_{50} value to be 372.2 μM and observed an anti-proliferative effect upon metronomic treatment with TMZ, which markedly inhibited HUVEC proliferation compared to conventional treatment of TMZ. In addition, since our metronomic treatment of TMZ was performed consecutively every 12 h, not daily, the IC_{50} value for growth inhibition of HUVECs in the present study differed from other reports. Thus, the discrepancy regarding the *in vitro* IC_{50} value and beneficial dose may be due to the experimental condition, including cell batch number, culture medium and treatment method. Our previous study found an anti-proliferative effect in HUVECs similar to the present study, in which no inhibitory effect was detected in either conventional TMZ single treatment or the combination of TMZ and IFN-β on HUVEC proliferation, although inhibition of proliferation was noted in GBM cells (29). This result also suggests that metronomic treatment with TMZ exerts an improved anti-proliferative effect on HUVEC proliferation. Therefore, as mentioned above, metronomic treatment with TMZ is a more beneficial therapy to inhibit proliferation of endothelial cells by tumor angiogenesis.

In this study, MGMT expression was down-regulated by metronomic treatment of TMZ in HUVECs, but not by conventional treatment of TMZ. MGMT has been the focus of research concerning drug resistance since the molecule was found to be highly expressed in several types of tumors, including colon cancer, breast cancer, myeloma, pancreatic tumor and gliomas (30,31). Regarding resistance to TMZ, Natsume *et al* reported that down-regulated MGMT expression by IFN-β results in the sensitization of resistant glioma cells to TMZ (13). Moreover, it has been reported that methylation of the MGMT promoter in gliomas is associated with responsiveness to alkylating reagents (14). One clinical research study found that long-term administration of TMZ leads to significant and prolonged depletion of AGAT activity in peripheral blood mononuclear cells, which may enhance the anti-tumor activity of methylating agents (16). In accordance with the reports mentioned above, we observed a reduced expression level of MGMT in HUVECs upon metronomic treatment of TMZ. Intriguingly, we also observed that the MGMT expression level was still down-regulated in several passages of HUVECs, indicating that metronomic TMZ chemotherapy sensitizes HUVECs by down-regulating MGMT expression (data not shown).

In relation to other anti-angiogenic activities, our study also showed that migration and tube formation were generally decreased in HUVECs cultured with metronomic treatment of TMZ. This result indicates that the decreased MGMT expression level upon administration of consecutive low doses of TMZ improves anti-angiogenicity in endothelial cells (Figs. 3 and 4). Helmlinger *et al* proposed that autocrine endothelial VEGF contributes to the formation of blood vessels. Overexpression of dimethylarginine dimethylaminohydrolase 2 was found to increase VEGF mRNA expression and enhance tube formation (32). A recent study demonstrated that MGMT modulates GBM angiogenesis, and identified angiogenesis related-genes, including VEGF and its receptors in MGMT-positive GBM cells, demonstrating that VEGF receptors are differentially expressed according to MGMT status (15). Based on these findings, we hypothesized that reduced migration and tube formation in HUVECs may result from metronomic treatment of TMZ which leads to a decreased MGMT expression level, but also that metronomic treatment of TMZ may alter and reconstruct endogenous expression and secretion of VEGF and its receptors in HUVECs.

In conclusion, this is the first *in vitro* study to report that the MGMT expression level can be modulated by metronomic treatment of TMZ; accordingly, it resulted in the decrease in proliferation, cell migration and tube formation in endothelial cells. In the present study, we also observed that MGMT inactivity is necessary to maximize the beneficial effect of methylating agents. Clinical trials on metronomic chemotherapy suggest various conditions and evidence; however, the adequate dose of TMZ chemotherapy for patients with tumors must be determined to allow individualized drug effectiveness. Thus, a consecutive treatment and a much lower dose of TMZ may allow its use in combination with other drugs in order to increase the chemotherapeutic efficacy and reduce side effects in clinical trials.

Acknowledgements

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