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Potentiation of antiglioma effect with combined temozolomide and interferon-ß

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Abstract. Temozolomide (TMZ) is a DNA methylating agent that has shown promising antitumor activity against high grade glioma. Interferon-ß (IFN-ß) is known to have antiproliferative and antiangiogenic activities. The aim of this study was to elucidate whether an antiglioma effect could be potentiated by the combination of TMZ and IFN-ß. *In vitro*, the combination of these drugs suppressed the proliferative and migratory activities, as well as enhance of the apoptosis and cell cycle (S phase) arrest of U-87 cells more efficiently than TMZ or IFN-ß alone. IFN-ß exerted a potent inhibitory effect on the proliferation of human umbilical vein endothelial cells (HUVEC); however, no additive or synergistic effect was observed with the addition of TMZ. To determine *in vivo* effect, nude mice bearing intracerebral U-87 xenograft inoculation were treated with intraperitoneal administration of PBS, TMZ (15 mg/kg for 3 days), IFN-ß ($2x10^5$ IU for 15 days), and a TMZ + IFN-ß combination. The combined treatment (median 62.0±8.6 days, P=0.0005) was observed to significantly increase the survival of the animals compared to treatment with PBS (median 30.0±2.5 days), TMZ (median 41.0±3.5 days) or IFN-ß (mean 36.0±2.5 days). These results suggest that antiglioma activity can be enhanced by the combination of TMZ and IFN-ß, providing the possibility for a new strategy development in the management of malignant glioma.

Introduction

Malignant gliomas, the most common primary brain tumor, are very aggressive tumors with a dismal prognosis despite advances in surgery, radiation therapy and chemotherapy (1).

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Temozolomide (3,4-dihydro-3-methyl-4-oxoimidazo-[5,1-d]-1,2,3,5-tetrazin-8-carboxamide, TMZ) is a new alkylating agent used in therapy for malignant gliomas (2). TMZ is a small lipophilic molecule with a molecular weight of 194 Da; it crosses the blood-brain barrier effectively, and it can be administered orally (3). The cytotoxic activity associated with TMZ has been attributed to methylation of the O^6 position of guanine, although this lesion accounts for only a small percentage of total-DNA adducts (4). During DNA synthesis, O^6-methylguanine mispairs with thymine rather than cytosine. The non-Watson/Crick pairings activate the mismatch repair (MMR) mechanism, and in turn, excises thymidine; reinsertion of this base occurs again only during repair DNA synthesis. The futile cycles of the MMR intervention lead to growth arrest and/or induction of apoptosis (5,6). TMZ is a potent inhibitor of glioma cell growth and angiogenesis at non-toxic doses (2). Despite the substantial improvement in health-related quality-of-life (7,8), TMZ treatment resulted in a meaningful but minimal survival benefit by the combination with radiotherapy in malignant glioma patients (9). Thus, further studies are needed to improve its clinical efficacy.

The interferons (IFNs) are a family of natural glycoproteins that consist of IFN-α, -ß and -γ; it is well known that the IFNs control cell growth and differentiation (10), inhibit expression of oncogenes (11), and activate T lymphocytes, natural killer cells, and macrophages (12,13). IFN-ß is known to cause cytotoxicity and apoptosis as well as stimulate the activity of apoptosis-related genes against tumor cells (14). In addition, it has been reported that IFN-ß sensitizes glioma cells to TMZ via induction of p53 and attenuation of O^6-methylguanine-DNA methyltransferase (MGMT) *in vitro* (15). Also, several *in vitro* and *in vivo* studies (16-19) have demonstrated an antiangiogenic activity attributed to IFN-ß in tumors. Chronic systemic administration of IFN-α or IFN-ß has been observed to produce regression of vascular tumors including Kaposi sarcoma (20), pulmonary hemangiomatosis (21), and hemangiomas (22); with only a marginal benefit for malignant gliomas (23). It has been reported that the combination of IFN with chemotherapy drugs increases the antitumor effects (24-26). Recently, combined therapy using TMZ and IFN-α has been shown to be quite tolerable in patients with metastatic melanoma (27) and renal cell carcinoma (28). In the present study, we investigated the effects of combined TMZ and IFN-ß in U-87 orthotopic xenograft brain tumor models and the possible mechanisms involved. We found that this novel

strategy has important therapeutic implications that may significantly increase therapeutic efficacy.

Materials and methods

Glioma cell proliferation assay. Human glioma cells (U-87, U251, U373; American Type Culture Collection, Rockville, MD, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL Co., Grand Island, NY, USA) containing 5% fetal bovine serum (FBS, Gibco). MTT assay was used to examine cell proliferation. Briefly, cell suspension (U-87: $3x10^3$ cell/well, U251 and U373: $1x10^3$ cell/well) was made with 200 μl media and plated on 96-well cell culture plates. After 24 h, the cells were treated with control media, 50-250 μM TMZ (Schering-Plough, Kenilworth, NJ, USA) or 100-800 IU/ml recombinant human IFN-ß (Schering AG, Berlin, Germany) for 3 days and dose-response was analyzed. The cells were also treated with control media, 60 μM TMZ, 400 IU/ml IFN-ß or 60 μM TMZ + 400 IU/ml IFN-ß combinations for 1-5 days to examine the effects if the combinations. The cells were incubated with MTT solution (Sigma Chemical CO., Steinheim, Germany) and then the optical density was measured at 550 nm using an ELISA reader (Molecular Devices, San Francisco, CA, USA).

Human umbilical vein endothelial cell (HUVEC) proliferation assay. HUVECs were cultured in M199 containing 10% FBS and ECGS (Sigma, St. Louis, MO, USA). After treatment with control media, 60 μM TMZ, 400 IU/ml IFN-ß or 60 μM TMZ + 400 IU/ml IFN-ß for 1-5 days, HUVECs were incubated with MTS solution (Promega, Madison, WI, USA) and the optical density was measured at 490 nm using an ELISA reader.

Cell cycle analysis. U-87 cells from cultures were washed with PBS and fixed in 70% ethanol at -20˚C for 8 h. The centrifuged pellets were resuspended in 500 μl of propidium iodide (PI, 50 μg/ml) and RNase (50 μg/ml). The cells were incubated at room temperature for 30 min in the dark room. The PI fluorescence was measured on a linear scale using a FACScan flow cytometer (Becton-Dicknson, San Diego, CA, USA).

Cell apoptosis assay. U-87 cells were treated with TMZ (60, 120 and 240 μM), IFN-ß (400, 800 and 1200 IU/ml) and each combination for 72 h. After the treatment, the cells were collected by scraping and lysed in a solution containing 0.5% SDS, 50 mM EDTA, 0.5 mg/ml proteinase K and 0.5 mg/ml RNase A, and incubated at 37˚C for 1 h. After DNA purification, each test sample was resuspended in TE buffer and loaded onto 1.5% agarose gel. DNA fragmentation was assessed by DNA gel electrophoresis. To examine the apoptotic cells by fluorescence microscopy, the cells were fixed with 4% paraformaldehyde in PBS for 10 min, stained by Hoechst 33258 (2 μg/ml) for 1 h, and observed by fluorescence microscopy (AX70; Olympus Optical Co., Tokyo, Japan). Annexin V and 7-AAD staining was quantified using the annexin V-PE apoptosis Kit (BD Biosciences, San Diego, CA) according to the instructions of the manufacturer. Briefly, U-87 cells were trypsinized, pelleted by centrifugation, and resuspended in annexin V binding buffer. PE-conjugated annexin V and 7-AAD were added to cells and incubated for 15 min at room temperature in the dark. Analyses were done on a FACScan (Becton-Dicknson). The data were analyzed with CellQuest software (Becton-Dickinson).

Wound migration assay. To assess the migration activity of U-87 cells, the cells were plated at a density of $5x10^5$ cells/well onto 6-well plates. A blue tip was used to create scraped wounds down the middle of the confluent monolayers. Cells were treated with control media, 60 μM TMZ, 400 IU/ml IFN-ß or 60 μM TMZ + 400 IU/ml IFN-ß for 8 h and the distance of their migration was determined by microphotographs.

Animal experiment. A brain tumor animal model was prepared as described previously (29). Briefly, 6- to 8-week old athymic nude (*nu/nu*) mice (BALB/nu-c, Shizuoka, Japan) were housed in laminar-flow cabinets under specific-pathogen-free conditions. The animals were anesthetized by intraperitoneal (i.p.) injection of xylazine (Rompun; Cutter Laboratories, Shawnee, KS, USA) 12 mg/kg and ketamine (Ketalar; Parke-Davis & Co., Morris Plains, NJ, USA) 30 mg/kg. The mice were held in a stereotactic frame with an ear bar. U-87 cells ($2x10^5$) in a volume of 3 μl PBS was injected slowly into the brain (i.c.) with a Hamilton syringe. The mice were randomly divided into 4 groups (n=6 in each group). The mice of each group were treated with i.p. injection of PBS (control group), TMZ 15 mg/kg from day 1 after the tumor inoculation for 3 days, IFN-ß $2x10^5$ IU from day 2 for 15 days, or TMZ + IFN-ß combination (TMZ 15 mg/kg from day 1 for 3 days + IFN-ß $2x10^5$ IU from day 2 for 15 days), respectively. Survival, neurologic signs and weight were recorded.

Statistical analysis. Statistical analysis was carried out using the Student's t-test (two-tailed); survival curves and mean values were generated by the Kaplan-Meier product-limit estimate. Data are represented as the mean ± SE. Differences were considered statistically significant at $P<0.05$.

Results

Antiproliferative effect of TMZ and IFN-ß on glioma cells and HUVECs. TMZ and IFN-ß demonstrated marked antiproliferative activity on U-87 human glioma cells in a dose-dependent manner, respectively (Fig. 1). Similar inhibition patterns were observed for U251 and U373 cells (data not shown). From these results, TMZ of 60 μM and IFN-ß of 400 IU/ml, which were the lower concentrations than each IC_{50}, were chosen for the following *in vitro* combination experiments. As shown in Fig. 2A-C, the combination of TMZ and IFN-ß had an additive antiproliferative effects on U-87, U251 and U373 cells. On the other hand, IFN-ß exerted a potent inhibitory effect on HUVEC proliferation, but TMZ did not. Combination of TMZ and IFN-ß showed neither an additive nor synergistic effect on HUVEC proliferation (Fig. 2D).

U-87 cell cycle arrest by TMZ and IFN-ß combination. U-87 cells were treated with the drugs for 24, 48, 72 or 96 h, and then analyzed for the cell cycle distribution. No significant

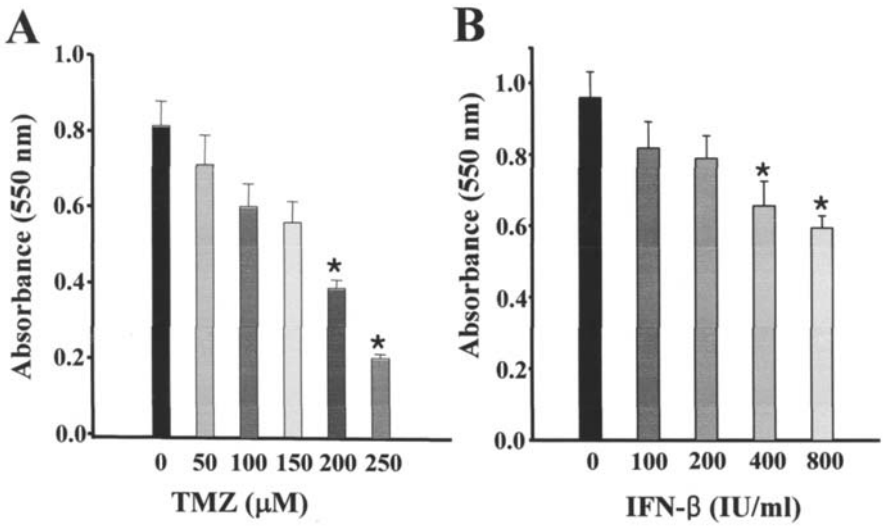


Figure 1. Antiproliferation effect of TMZ and IFN-ß. Human glioma cells were seeded into a 96-well plate and allowed to adhere overnight. Cells were cultured with control medium or medium containing TMZ or IFN-ß (*P<0.01). (A), TMZ monotherapy-dose response at 72 h. (B), IFN-ß monotherapy-dose response at 72 h.

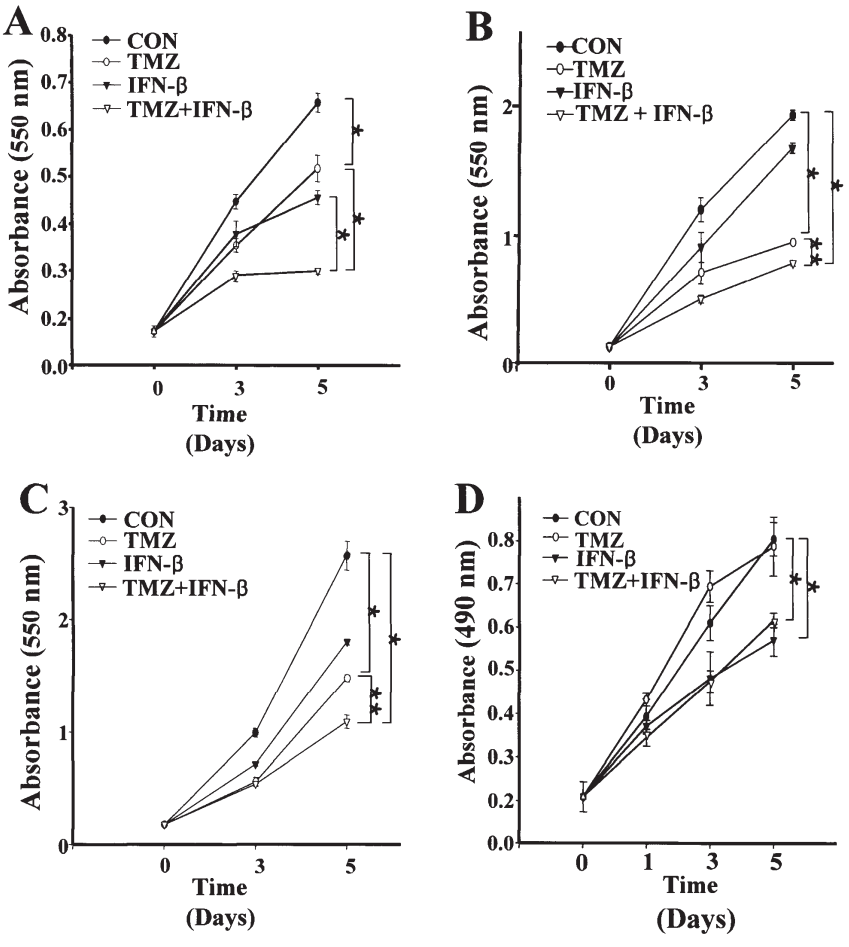


Figure 2. Antiproliferation effect of TMZ and IFN-ß on glioma cells and HUVECs. (A-C), U-87 (A), U251 (B) and U373 (C) cells were treated with TMZ (60 μM), IFN-ß (400 IU/ml) or TMZ (60 μM) + IFN-ß (400 IU/ml) from day 1 to 5. (D), HUVECs were seeded into 96-well plate and allowed to adhere overnight. Cells were cultured with control medium or medium containing TMZ (60 μM), IFN-ß (400 IU/ml) or TMZ (60 μM) + IFN-ß (400 IU/ml) from day 1 to 5. Live cells were measured using MTT or MTS assay. *P<0.01; **P<0.01 on 5 days.

change was observed in the cell cycle regulation with any treatment at 24 h (data not shown). As shown in Fig. 3, TMZ and IFN-ß alone did not induce a cell cycle arrest, but the combination of TMZ and IFN-ß was associated with S phase

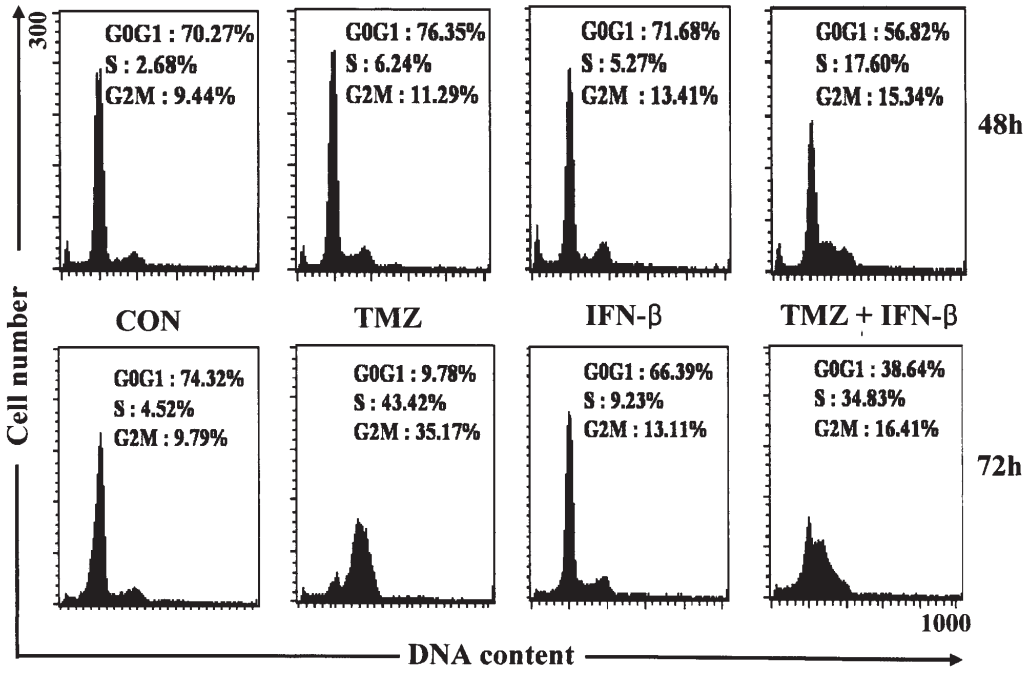
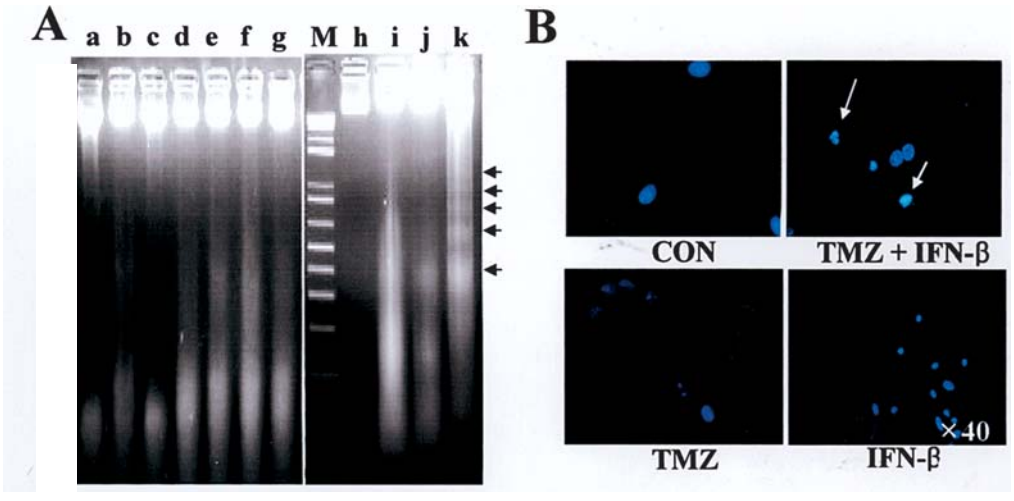


Figure 3. Induction of cell cycle arrest by TMZ and IFN-ß. U87 cells were treated with TMZ (60 μM), IFN-ß (400 IU/ml) or TMZ + IFN-ß combination for 48 h (upper panel) or 72 h (lower panel). PI-stained cells were analyzed by means of FACScan.



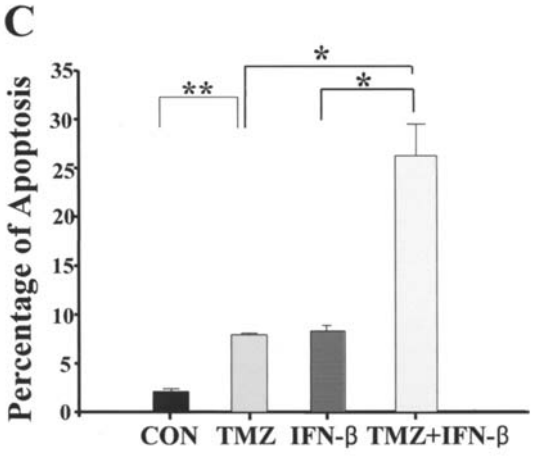


Figure 4. Apoptosis induction by TMZ and IFN-ß. (A), After treatment of U-87 cells with TMZ and IFN-ß for 72 h, fragmented DNA was extracted from the cells and electrophoresed. (Lanes: a, control; b, 60 μM TMZ; c, 400 IU/ml IFN-ß; d, 60 μM TMZ + 400 IU/ml IFN-ß; e, 120 μM TMZ; f, 800 IU/ml IFN-ß; g, 120 μM TMZ + 800 IU/ml IFN-ß; M, DNA marker; h, control; i, 240 μM TMZ; j, 1200 IU/ml IFN-ß; k, 240 μM TMZ + 1200 IU/ml IFN-ß). Arrows indicate fragmented DNA. (B), The fluorescent microphotograph showing DNA condensation of the nucleus in U-87 cells at 72 h after treatment with TMZ and IFN-ß. Fluorescence microscopy dye, Hoechest 33258, showed chromatin condensation and apoptotic bodies (arrows). (C), FACScan analysis of U-87 cell apoptosis using annexin V-PE and 7-AAD staining. TMZ 240 μM and IFN-ß 1200 IU/ml induced 7.9 and 8.3% of cells to undergo apoptosis. The combined treatment increased the number of apoptotic cells (26.2%). *P<0.03; **P<0.01.

arrest at 48 h. TMZ increased the number of cells in the S phase and G_2M phase, and IFN-ß did not influence the cell cycle, however, the combined treatment induced cell cycle arrest in the G_1 phase and the S Phase in a pattern different from TMZ treatment alone at 72 h. TMZ, IFN-ß, and their combination induced cell cycle arrest at 96 h.

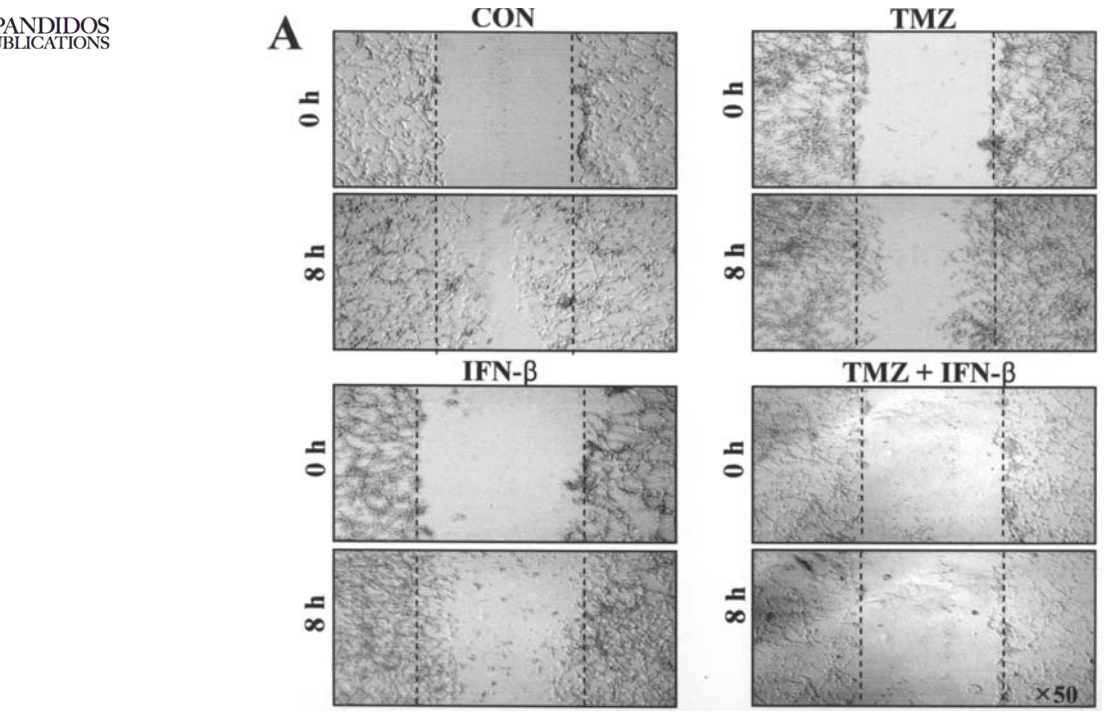


Figure 5. Antimigration effect of TMZ and IFN-ß. (A), Photomicrographs showing migration of U-87 cells after 8 h in the absence and treatment of TMZ (60 μM) or IFN-ß (400 IU/ml). (B), A graphical presentation of cell migration. The data are represented as the distance of migrated cells treated with TMZ and IFN-ß, $^{*}P<0.03$; $^{**}P<0.01$.

Enhanced apoptosis in U-87 cells by TMZ and IFN-ß combination. In addition to their cell growth and cell cycle checkpoint effects, we investigated the combined effects on apoptosis. U-87 cells were treated with TMZ (60, 120 and 240 μM), IFN-ß (400, 800 and 1200 IU/ml) and each combination. As shown in Fig. 4A, gel electrophoresis exhibited DNA ladder formation only in the cells treated with the combination of 240 μM TMZ and 1200 IU/ml IFN-ß at 72 h (Fig. 4A, h). The U-87 cells in control and 240 μM TMZ (Fig. 4A, i) or 1200 IU/ml IFN-ß group (Fig. 4A, j) showed no morphological changes detected under similar conditions. These cells also displayed the nuclear morphological changes characteristic of apoptosis under the fluorescent microscope (Fig. 4B). We observed condensational U-87 cells in only the 240 μM TMZ + 1200 IU/ml IFN-ß combination group. These findings suggest that the combination approach may be a better strategy for the induction of apoptosis than a monotherapy, even though a high concentration of TMZ and IFN-ß is required. Furthermore, when the apoptotic rates of U-87 cells after TMZ and IFN-ß treatment were quantified by annexin V and 7-AAD double staining followed by flow cytometry analysis (annexin V-positive and 7-AAD negative cells were interpreted as early apoptotic cells), the TMZ and IFN-ß combination treatment increased the number of apoptotic cells (26%) compared to the single treatments (Fig. 4C).

Suppression of U-87 cell migration by TMZ and IFN-ß combination. We also investigated the effect of TMZ and IFN-ß on U-87 cell migration using a wound migration assay. The observations showed that either TMZ (60 μM) or IFN-ß (400 IU/ml) suppressed the migratory activity of U-87 cells, and that the suppression of U87 cell migration was enhanced by the combination of TMZ and IFN-ß (Fig. 5).

Survival of tumor-bearing animals treated with TMZ and IFN-ß combination. Control mice that received i.p. injections of PBS died after development of large brain tumors 25-36 days after tumor implantation. The animals treated with either TMZ or IFN-ß alone, survived for 33-45 days ($P<0.05$) and 32-41 days ($P<0.05$), respectively. By contrast, the animals treated with the combined TMZ and IFN-ß survived for 49-80 days ($P=0.0005$). Therefore, the combination treatment significantly increased survival of the brain tumor-bearing mice, compared to the monotherapy group (Fig. 6).

Discussion

TMZ has been widely used for the treatment of adult patients with anaplastic astrocytoma and glioblastoma patients in the United States and the European Union (30). Despite its therapeutic limitations, many studies have demonstrated the antitumor effect of TMZ could be improved with combination of

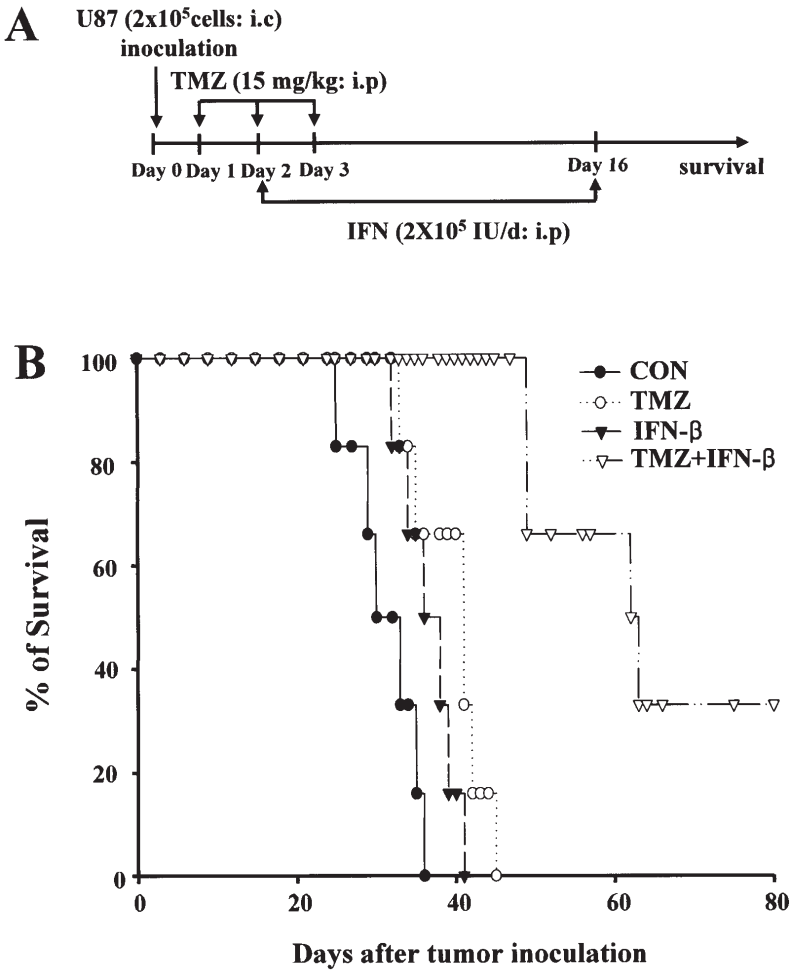


Figure 6. TMZ and IFN-ß, combination therapy prolongs the survival of U-87 brain tumor-xenografted mice. (A), The experimental design of survival study. Daily intraperitoneal injections of TMZ (15 mg/kg/day) were performed 1-3 days after the tumor inoculation. Administration of IFN-ß ($2x10^5$ IU/day) i.p. were performed 2-16 days. For the combination treatment, TMZ treatment followed by IFN-ß treatment were performed by the same dose and time schedule. (B), Survival of tumor-bearing mice treated with TMZ and IFN-ß.

radiation or other chemotherapeutic drugs (9,31-33). IFN-ß has also been used for patients with solid tumors including malignant gliomas with only marginal benefit; a therapeutic guideline for optimal clinical efficacy has not been determined (23,34). It has been reported that the antitumor effect of IFN-ß can be improved through the combination with chemotherapy and radiation therapy (26).

In the present study, we investigated whether antiglioma activity could be potentiated by the combination of TMZ and IFN-ß. Each of these drugs has been used for the treatment of patients with malignant glioma. The combination treatment strategy was observed to suppress the proliferative and migratory activities of U-87 cells, enhance apoptosis and cell cycle (S phase) arrest, and increased survival of the tumor-bearing animals significantly compared to animals treated with TMZ or IFN-ß alone.

In regard to the mechanism for cellular action, TMZ is known to be a potent inhibitor of glioma cell growth and angiogenesis via methylation of the O^6 position of guanine and the futile cycles of MMR intervention (2-6). IFN-ß was reported to control the cell growth and differentiation, cause cytotoxicity and apoptosis (11,14), and inhibit angiogenesis in tumors (16-19,35). In our study, the proliferation of U-87, U251 and U373 cells was inhibited by TMZ and IFN-ß, and this inhibition was enhanced when the two were combined. Since U-87 cells have wild-type p53, and U251 and U373 cells have mutant type p53, our results suggest that antiglioma effect of the combined therapy may not be influenced by the p53 status of the glioma. TMZ was reported to be a potent inhibitor of angiogenesis at non-toxic doses in CAM assay (2). IFN-ß inhibits HUVEC proliferation (35), however, in our assay, the proliferation of HUVEC was not inhibited by TMZ at the same dose and the inhibitory effect on the HUVEC proliferation was not potentiated by the combination of TMZ and IFN-ß. This result suggests that IFN-ß might have a helper effect through the inhibition of angiogenesis and enhancement of antiproliferative activity of TMZ against the brain tumor *in vivo*.

Next, we addressed if increased growth inhibition effect of TMZ and IFN-ß combination might be accompanied by modulation of cell cycle progression. Growth suppression of tumor cells by chemopreventive or chemotherapeutic agents including TMZ is known to be associated with blocking the cell cycle progression at S, G_2M phase checkpoint (36). In addition, Chk1, cdc2 and p38 pathways cooperate to bring about TMZ-induced G_2 arrest (37,38). Arrest of the G_1 and

the main cell cycle manifestation of IFN-ß (23) and ves U-87 cells into a senescence-like state (39). In the present study, we found that the cell cycle arrest was caused by TMZ, IFN-ß and TMZ+IFN-ß combination at 96 h, by TMZ and TMZ+IFN-ß combination at 72 h, and only by TMZ+IFN-ß combination at 48 h. This indicates that earlier induction of cell cycle arrest can be achieved by the combination of TMZ and IFN-ß.

And G_2M arrest induced by TMZ was clearly detected, however, we identified an increase of G_1 phase again due to combined TMZ and IFN-ß treatment at 72 h. Our findings suggest the possibility that the cell cycle may progress as a result of IFN-ß or that DNA alteration may occur upon treatment with combination therapy. The molecular alterations underlying the cell cycle arrest induced by combined TMZ and IFN-ß remains to be determined.

Since it has been reported that TMZ (5,40) and IFN-ß (41) induce apoptosis in glioma cells, we performed a dose-dependent fragmentation assay to determine the apoptosis that results from combined TMZ and IFN-ß in U-87 cells. Apoptosis was not observed when U-87 cells were treated with TMZ at a dose of 60 μM and/or IFN-ß at a dose of 400 IU/ml. Apoptosis was induced by the combined treatment with TMZ at a dose of 240 μM and IFN-ß at a dose of 1200 IU/ml. A combined TMZ and IFN-ß treatment significantly increased DNA fragmentation, DNA condensation, apoptotic cells, which indicates an increase of direct apoptotic effects. Increased apoptosis may be one of the important mechanisms related to the enhanced *in vivo* effect of TMZ and IFN-ß combination.

Malignant glioma cells are characterized by rapid cell proliferation, high motility, and invasiveness. These important biologic characteristics are related to the malignant behavior of this tumor. It has been reported that the migratory behavior of glioblastoma cells is diminished by IFNs (42,43). In addition, U-87 tumor spheroids treated with TMZ, are reported to show a reduction in the invasion velocity (40). In our wound migration assay, each of TMZ and IFN-ß decreased the migration activity of U-87 cells directly and their combination synergistically suppressed the migratory activity. Markedly diminished migratory activity of U-87 cells might be another explanation for the survival prolongation of the animals by the combination approach.

In our preliminary *in vivo* experiments, i.p. administration of TMZ at doses of 15, 30 or 50 mg/kg for 3 days resulted in a dose-dependent survival increase (data not shown), and we used TMZ dose of 15 mg/kg for the following experiments. We have previously reported that IFN-ß $2x10^5$ IU suppresses the *in vivo* U-87 glioma growth more efficiently at early stage than at late stage of the tumor development (35). In the present study, we designed an *in vivo* experiment to investigate the effect of the combined treatment on the survival of the tumor-bearing animals at an earlier stage of the tumor development, and treated the animals with i.p. administration of TMZ from day 1 for 3 days and IFN-ß from day 2 for 15 days after the tumor inoculation. The combination therapy significantly increased the survival of the tumor-bearing mice compared to the monotherapy group.

IFNs have been known to inhibit angiogenesis not only by affecting endothelial cells but also by inhibiting production or release of factors from tumor cells or lymphocytes which may stimulate the host response (35) and regulates NK cell cytotoxicity through the STAT1 pathway (44). This could be another reason for the potentiation of the *in vivo* antitumor effect by the combined therapy in our study. In conclusion, the combination of TMZ and IFN-ß may be suggested as a valuable strategy to be developed into a new treatment modality for the malignant glioma patients with a minimal tumor burden.

Acknowledgements

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