Effect of IFN-ß on human glioma cell lines with temozolomide resistance

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Abstract. Human interferon-ß (IFN-ß) is known to exhibit pleiotropic biological activities including antitumor effects. On the other hand, temozolomide (TMZ), an oral bioavailable alkylating agent with excellent tolerability, has demonstrated efficacy and has become a key therapeutic agent in patients with malignant gliomas; however, its survival benefit remains unsatisfactory. More recent studies have indicated that there might be favorable therapeutic interactions between IFN-ß and TMZ, although the therapeutic advantages of such a combination have not yet been fully explored. The main aim of the present study was to determine whether an antitumor effect could be potentiated by a combination of IFN-ß and TMZ. The antitumor effect of and cell sensitivity to IFN-ß and TMZ and the synergistic potential of IFN-ß and TMZ in combination were evaluated in six malignant glioma cell lines. Correlations among the *MGMT* methylation status, quantitative level of *MGMT* mRNA, MGMT protein expression and the antitumor effect of these agents were also evaluated, since one of the most prominent resistance mechanisms to TMZ involves the DNA repair protein MGMT. The cell growth inhibitory effects of IFN-ß and TMZ on all tumor cell lines were observed in a dose-dependent manner, and the human malignant glioma-derived cell lines differed in their sensitivity to TMZ. The MGMT status, including promoter hypermethylation, quantitative mRNA expression and protein expression, was strongly correlated with TMZ sensitivity. A synergistic cell growth inhibitory effect and down-regulated *MGMT* mRNA levels were significantly observed when a clinically achievable CNS dose of IFN-ß was combined with TMZ, as compared to treatment with IFN-ß or TMZ alone in TMZ-resistant T98G cells. Furthermore, significant amounts of endogenous IFN-ß protein were detected in TMZ-treated T98G cells by ELISA. These results suggest that the clinical therapeutic efficacy of TMZ might be improved by a combination with IFN-ß in malignant gliomas unmethylated at the *MGMT* gene. The data provide an experimental basis for future strategies in TMZ chemotherapy, although further studies are needed to determine the detailed role of combined IFN-ß and TMZ chemotherapy in increasing tumor sensitivity.

Introduction

Malignant gliomas are the most common primary tumors of the central nervous system. They continue to present an enormous therapeutic challenge as multimodality treatments including extensive tumor resection, radiation therapy and chemotherapy have contributed little improvement to the poor prognosis over the last three decades.

Although there is an increasing diversity of drugs available for chemotherapy, alkylating agents have been widely used alone or in combination with other drugs to treat malignant gliomas. Temozolomide (3-methyl-4-oxo-3,4-dihydro-imidazo [5,1-*d*] [1,2,3,5] tetrazine-8-carboxamide) (TMZ), a relatively new alkylating (methylating) agent, has received much attention, notably in the treatment of malignant gliomas. Phase II and III trials for recurrent high-grade gliomas have revealed that 50 to 60% of glioblastomas, the most malignant form, showed an objective response or stable disease with this compound (1-4). A more recent European Organisation for Research and Treatment of Cancer/National Cancer Institute of Canada trial on concomitant and adjuvant TMZ in addition to radiotherapy as the first-line treatment for glioblastomas demonstrated an increase in median survival from 12.1 to 14.6 months and an increase in the 2-year survival rate from 10 to 26% as compared to radiotherapy alone (5). The cytotoxicity of TMZ is thought to be due primarily to the formation of O^6-methylguanine in DNA, which mispairs with thymine during the next cycle of DNA replication (6). Subsequent futile cell cycles of DNA mismatch repair lead to growth arrest and/or induction of apoptosis (7,8). On the other hand, the suicide DNA repair enzyme, O^6-methyl-

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guanine-DNA methyltransferase (MGMT), reverses alkylation at the O^6-position of guanine (9,10). One of the key factors controlling sensitivity to O^6 alkylating agents including TMZ in tumors is therefore considered to be the action of MGMT (10-13). Indeed, several investigators have reported that, in malignant gliomas, MGMT expression is closely correlated with experimental and clinical resistance to these chemotherapeutic agents (14-18). Furthermore, recent studies have indicated that, since MGMT protein loss may be a result of promoter hypermethylation, methylation of the *MGMT* promoter is significantly correlated with clinical outcome in malignant glioma patients treated with alkylating agents including TMZ (19-29).

Human interferon-ß (IFN-ß), which belongs to the type I IFNs, was first discovered on the basis of its antiviral activities. Subsequently, it was shown to exhibit pleiotropic biological activities including immunomodulatory activity, anti-angiogenetic activity and direct antitumor effects, e.g. growth inhibition and apoptosis (30-32). In addition to such multiple functions of IFN-ß against human neoplasias, it acts as a drug sensitizer enhancing the toxicity against a variety of neoplasias when administered in combination with nitrosoureas (alkylating agents) (33). It is therefore widely employed either alone or in combination with other antitumor agents such as nitrosoureas in the treatment of gliomas, particularly in Japan (30,33-35). More recently, a synergistic antiproliferative effect between TMZ and IFN-ß was demonstrated in malignant glioma cells *in vitro* (36,37). Notably, Natsume *et al* (36) reported that the sensitizing effect of IFN-ß in TMZ-resistant glioma cells is possibly due to an attenuation of MGMT expression via induction of the protein p53. These findings suggest that favorable therapeutic interactions might take place between IFN-ß and TMZ, although the concentrations of both agents used in these experiments were considered to exceed the values of clinically achievable concentrations.

As mentioned above, concomitant radiation therapy and chemotherapy with TMZ followed by adjuvant TMZ treatment can yield meaningful results, but the survival benefit in malignant glioma patients is minimal (5). Thus, further studies are needed to improve the clinical therapeutic efficacy and to establish a therapeutic strategy to circumvent MGMT-mediated resistance to TMZ. It is important to assess whether or not a synergistic effect operates between IFN-ß and TMZ in TMZ-resistant malignant glioma cells, particularly at clinically relevant concentrations of IFN-ß and TMZ and to evaluate the mechanism and establish an experimental basis for rational clinical combination therapy with IFN-ß and TMZ.

IFNs are produced by organisms (most cells are believed to be capable of type I IFN production) in response to various stimuli. Since polyribonucleotides were discovered to induce high levels of serum IFN in 1967, several additional low-molecular-weight inducers including tilorone, pyrimidinones, acridines and imidazoquinolinamines have been described (38,39). The search for effective inducers, particularly those which might be orally active, has attracted much attention (38). On the other hand, IFN-ß gene therapy research, for example the series of Yoshida and his coworkers (40-46), revealed that the endogenous IFN-ß protein produced by tumor cells inhibits tumor cell growth much more strongly than does exogenously added IFN-ß protein in malignant gliomas. However, little is known regarding whether or not TMZ, a low-molecular-weight and oral bioavailable alkylating agent, is able to induce malignant glioma cells to produce endogenous IFN-ß protein. In the present study, we therefore investigated whether malignant glioma cells can produce endogenous IFN-ß protein by exposure to TMZ.

Materials and methods

Tumor cell lines. Human malignant glioma U-87MG and U-138MG cells were purchased from the American Tissue Culture Collection (ATCC; Manassas, VA, USA), and A-172, AM-38, T98G and U-251MG cells were purchased from the Health Science Research Resources Bank (Sennan, Osaka, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco™, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco) in a standard humidified incubator at 37˚C in a 5% CO_2-95% air atmosphere.

Detection of MGMT methylation. Promoter hypermethylation of the *MGMT* gene was determined by the methylation-specific polymerase chain reaction (MS-PCR) (47). DNA was extracted using a QIAamp® DNA Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. The methylation status of the CpG islands of the *MGMT* gene was determined by chemical modification of unmethylated, but not the methylated, cytosine to uracil. The subsequent PCR was performed with primers specific for either methylated or modified unmethylated DNA, as described previously (22). Briefly, 1 μg of extracted DNA underwent bisulfite modification using a CpGenome™ DNA Modification Kit (Chemicon™, Millipore Corp., Billerica, MA, USA), according to the manufacturer's instructions. The primer sequences of *MGMT* for the methylated and unmethylated reactions were as reported previously (22). The PCR was carried out as described previously (48,49). Amplified products were electrophoresed on 3% agarose gels and were visualized with ethidium bromide. Enzymatically methylated human male genomic DNA (CpGenome Universal Methylated DNA; Chemicon, Millipore Corp.) and DNA from normal lymphocytes were included in each set of the PCR as methylated and unmethylated controls, respectively.

Quantitation of MGMT mRNA by real-time quantitative RT-PCR. Quantitation of *MGMT* gene expression was performed by the real-time quantitative reverse transcription-PCR (RT-PCR) method, as developed recently (18,50). Briefly, complementary DNA was synthesized from 1 μg total RNA with a random primer (Invitrogen, CA, USA), 40 U M-MLV reverse transcriptase (Invitrogen), 0.5 mM dNTP (Takara Bio, Shiga, Japan), 24 U RNase inhibitor (Takara Bio), 10 μM DTT (Invitrogen) and 5X RT buffer at 37˚C for 60 min. The real-time PCR reaction mixture was prepared using a TaqMan Universal Master Mix (Applied Biosystems, CA, USA), 120 nM of each primer (MGMT forward primer 5'-CCT GGC TGA ATG CCT ATT TC-3' and reverse primer 5'-GAT GAG GAT GGG GAC AGG ATT-3'), 200 nM probe

(5'-CGA GCA GTG GGA GGA GCA ATG AGA-3') and 2.5 μl of each cDNA sample. The PCR conditions were as follows: 1st stage, 95˚C for 10 min; 2nd stage, 45 cycles at 95˚C for 30 sec, 60˚C for 30 sec, 72˚C for 30 sec; and 3rd stage, 72˚C for 10 sec, using a real-time PCR system (ABI PRISM 7900HT Sequence Detection System; Applied Biosystems). *Glyceraldehyde-3-phosphatase dehydrogenase* (*GAPDH*) mRNA expression levels were employed as the quantitative internal control. Standard curves for *MGMT* and *GAPDH* mRNA were generated using 10-fold serially diluted standard plasmid clones inserted by MGMT or GAPDH PCR products as templates, and the amount of each mRNA expression level was calculated from the relevant standard curve. For precise quantification, the *MGMT* mRNA expression level of each sample was normalized using the expression of the *GAPDH* gene.

Western blot analysis of MGMT. To determine the protein levels of MGMT, soluble protein lysates of subconfluent glioma cells were obtained employing lysis buffer (Medical & Biological Laboratories, Woburn, MA, USA) for 20 min on ice. The proteins (5-μg portions) were loaded and separated by 12.5% polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Franklin Lakes, NJ, USA) for 30 min at 10 V with a Bio-Rad transblot (Bio-Rad Laboratories). Non-specific binding was blocked with a wash buffer (PBS/0.05% Tween-20) containing 5% ECL blocking agent (GE Healthcare UK Ltd., Amersham Place, Little Chalfont, Buckinghamshire, UK) for 60 min at room temperature. The primary antibodies and dilutions used for the immunoblotting were MGMT (1:200) (MT 3.1; Neomarkers, Fremont, CA, USA) for 120 min and ß-actin (1:5000) (AC-15; Sigma-Aldrich, St. Louis, MO, USA) for 60 min. The secondary antibodies employed were bio-tinylated anti-mouse IgG (H and L) (1:1000) (BA-2000; Vector Laboratories, Burlingame, CA, USA) for 60 min. The immunoblotted complex was visualized with the aid of the ECL Western blotting analysis system (GE Healthcare UK Ltd.).

Cell culture growth studies with IFN-ß or TMZ

Growth inhibitory effect. The growth inhibition of the malignant glioma cells by IFN-ß or TMZ was evaluated by counting the numbers of cells. Briefly, cells were plated at 1x10⁴ or 2x10⁴ cells per well in 24-well, flat-bottomed plates and incubated with medium for 24 h. The cells were subsequently washed twice with medium and further incubated with fresh medium (control), medium containing 0.1-1000 IU/ml of IFN-ß or medium containing 0.1-1000 μM of TMZ. After exposure to the various concentrations of IFN-ß or TMZ for 72 h, cells were detached by trypsinization and counted. The experiments were repeated at least four times at each concentration.

Cell death effect. The *in vitro* cell death effect of IFN-ß or TMZ on the glioma cell lines was evaluated by counting the floating cells in the media. Briefly, cells were plated at 1-3x10⁵ cells per well in 24-well plates and incubated with medium for 24 h. After exposure to the various concentrations of IFN-ß or TMZ for 72 h, floating cells were collected and counted using trypan blue dye. The experiments were repeated at least four times at each concentration.

Studies on the enhanced effects of TMZ by IFN-ß

Growth inhibitory effect of IFN-ß and TMZ. The TMZ incubation condition (10 μM of TMZ) was chosen since this level of TMZ demonstrated growth inhibition in cell lines not showing MGMT expression (A-172, AM-38, U-87MG and U-251MG), but not in cell lines with MGMT expression (T98G and U-138MG). Furthermore, this condition represents a clinically relevant concentration of TMZ (51).

Glioma cells were plated and incubated with medium for 24 h and then incubated with medium containing various concentrations (0-1000 IU/ml) of IFN-ß with 10 μM TMZ or without TMZ. After exposure to the various concentrations of IFN-ß with 10 μM TMZ or without TMZ for 72 h, cells were detached by trypsinization and counted. The experiments were repeated at least four times at each concentration.

Combined effect of TMZ and IFN-ß in TMZ-resistant cells. To further examine the influence of TMZ and IFN-ß in glioma cell lines, particularly TMZ-resistant cells, we chose the T98G cell line which demonstrates promoter unmethylated *MGMT* together with MGMT expression by Western blot analysis and quantitative levels of mRNA.

T98G cells were plated at 1x10⁴ cells per well in 24-well flat-bottomed plates and incubated with medium for 24 h. They were then exposed to the following four conditions: medium only (control), medium with 10 IU/ml IFN-ß, 10 μM TMZ, or a combination of both 10 IU/ml IFN-ß and 10 μM TMZ for 24 or 48 h. These conditions also represent clinically relevant concentrations of IFN-ß and TMZ (51,52). After exposure to such conditions, cells were detached by trypsinization and counted. The experiments were repeated at least four times at each concentration.

Furthermore, to evaluate the hypothesis that IFN-ß might increase TMZ toxicity due, at least in part, to decreased *MGMT* expression, T98G cells were exposed to the following four conditions: control, 10 IU/ml IFN-ß, 10 μM TMZ, or a combination of 10 IU/ml IFN-ß and 10 μM TMZ for 6 h, after which the quantitative levels of *MGMT* mRNA were assayed using real-time quantitative PCR at least five times under each condition.

Quantifications of endogenous IFN-ß protein production. T98G cells (1x10⁶) were plated in 25-cm² flasks and cultured in DMEM supplemented with 1% fetal bovine serum (total 5 ml) in a standard humidified incubator at 37˚C in a 5% CO_2-95% air atmosphere for 24 h, and the cells were then further incubated with or without 10 μM TMZ. After incubation with or without 10 μM TMZ for 24, 48 or 72 h, the cell culture supernatants were collected and stored at -80˚C until the time of IFN-ß protein assay. The quantities of IFN-ß protein were determined with the enzyme-linked immunosorbent assay (ELISA) system established in Toray Industries (53). This ELISA correlated well with a bioassay calibrated against the international reference standard for IFN-ß, confirming that this ELISA could quantify biologically active IFN-ß molecules (53). The experiments were repeated at least six times at each point.

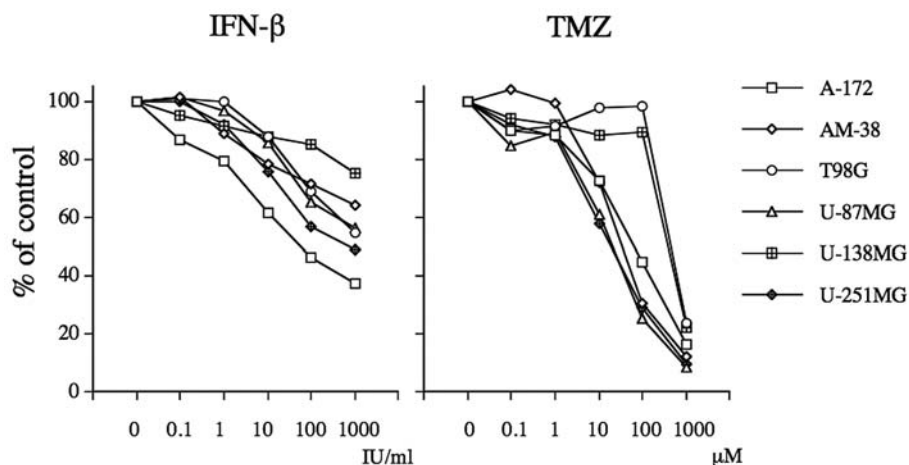Figure 1. Antitumor effects of IFN-ß (left) and TMZ (right) against six human glioma cell lines. At 72 h after the addition of IFN-ß (final concentration, 0-1000 IU/ml) or TMZ (final concentration, 0-1000 μM) to the culture medium, the number of viable cells was counted. The number was expressed as a percentage of the untreated control.
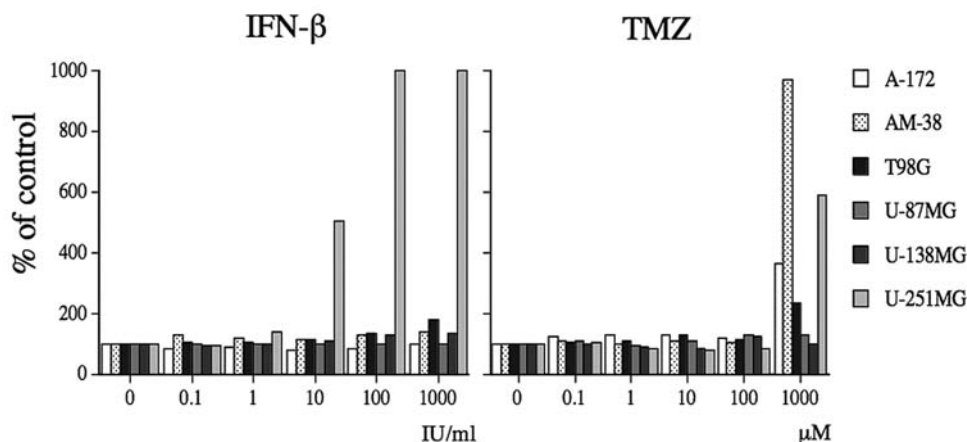

Figure 2. No significant increase in the percentage of trypan blue-positive floating cells in the media occurred after 72 h of IFN-ß treatment (0-1000 IU/ml) except in U-251MG cells. On the other hand, TMZ did not induce any increase in the percentage of trypan blue-positive floating cells at any concentration up to 100 μM.

Results

Antitumor effect of and cell sensitivity to IFN-ß and TMZ in human malignant glioma cells. To determine the antitumor effects of IFN-ß or TMZ alone in malignant glioma cells, we treated six malignant glioma cell lines with 0-1000 IU/ml of IFN-ß or 0-1000 μM of TMZ for 72 h and assessed the numbers of viable cells. As shown in Fig. 1, the cell growth inhibitory effects of IFN-ß and TMZ on all tumor cell lines were observed in a dose-dependent manner. The 50 percent inhibition concentration (IC_{50}) of IFN-ß for A-172 and U-251MG was <100 IU/ml and ~1000 IU/ml, respectively. The IC_{50} values of the other four cell lines were >1000 IU/ ml. On the other hand, the IC_{50} of TMZ for four malignant glioma cells (A-172, AM-38, U-87MG and U-251MG) was <100 μM, although T98G and U-138MG cells were found to be resistant to TMZ (IC_{50} >100 μM).

On the basis of the hypothesis that part of the antigrowth effects of IFN-ß and TMZ might be a result of cytocide, the six malignant glioma cell lines were treated with IFN-ß or TMZ and assessed by counting the trypan blue-positive floating cells in the media. No significant increase in the percentage of cell staining with trypan blue dye occurred after 72 h of IFN-ß (0-1000 IU/ml) treatment except in U-251MG cells. In these cells, dose-response studies at 72 h demonstrated greater numbers of trypan blue-positive cells with increasing doses of IFN-ß (Fig. 2). The effect of IFN-ß was thus cytocidal only in U-251MG cells and was cytostatic in the other five human malignant glioma cell lines. On the other hand, TMZ did not induce any increases in the percentage of cell staining with trypan blue at any concentration up to 100 μM, indicating a cytostatic rather than cytocidal action on all the six human glioma cell lines.

Correlations of MGMT methylation status, quantitative MGMT mRNA expression and MGMT protein expression with TMZ sensitivity. Methylation of the CpG islands in promoter regions is a major factor in gene regulation and is
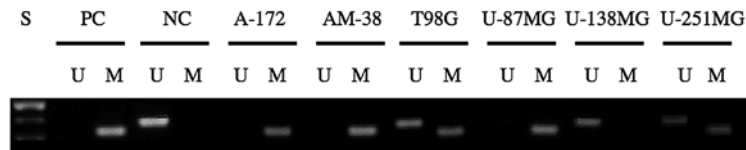
Figure 3. Methylation-specific PCR of the *MGMT* promoter in six glioma cell lines. Methylated DNA was evident in A-172, AM-38 and U-87MG. The U-138MG cell line showed unmethylated DNA. The other two cell lines, T98G and U-251MG, appeared to contain methylated and unmethylated DNA at this locus. S, molecular size marker; U, PCR product amplified by unmethylated-specific primer; M, PCR product amplified by methylated-specific primer; PC, positive control; NC, normal control.
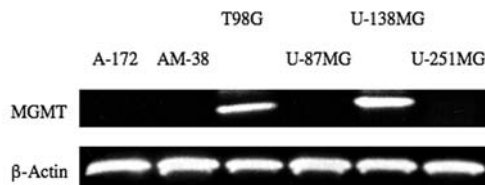

Figure 4. Western blot analysis of MGMT expression at the protein level in six human glioma cell lines. This revealed an absence of MGMT expression in the four cell lines A-172, AM-38, U-87MG and U-251MG, all of which failed to show detectable *MGMT* mRNA by real-time quantitative RT-PCR. In contrast, the other two cell lines, T98G and U-138MG, revealed MGMT expression at the protein level, with both of them showing detectable *MGMT* mRNA by real-time quantitative RT-PCR. ß-actin was used as a loading control. Data are representative of four experiments.

correlated with transcriptional silencing. To confirm the *MGMT* methylation status, DNA from the six malignant glioma cell lines was isolated and investigated by performing MS-PCR. Furthermore, in order to determine whether or not MGMT expression might be responsible for part of the resistance mechanism to IFN-ß and TMZ in glioma cells, we tested the MGMT expression at the mRNA and protein levels.

Several of our findings corroborate those of a recent study (54). Promoter hypermethylation of *MGMT* was demonstrated in A-172, AM-38 and U-87MG (TMZ-sensitive cell lines) but not in the U-138MG cell line (TMZ-resistant cell line). The other two cell lines, T98G and U-251MG, appeared to contain methylated and unmethylated DNA at this locus (Fig. 3). Furthermore, MGMT expression as determined from

MGMT mRNA expression levels using real-time quantitative RT-PCR and Western blot analysis was not detected in the TMZ-sensitive cell lines A-172, AM-38, U-87MG and U-251MG (Fig. 4). In contrast, such expression was detected in the TMZ-resistant cell lines T98G and U-138MG. The absolute values of *MGMT* mRNA normalized to the level of *GAPDH* in T98G and U-138MG were $5.9x10^3$ and $6.3x10^3$ copy/μg RNA, respectively (Table I). These findings indicated that the resistance of T98G and U-138MG cells to TMZ was probably related to MGMT.

Antitumor effects of a combination of IFN-ß and TMZ. To assess whether or not a combination of TMZ and IFN-ß could produce an additive antitumor effect in the six malignant glioma cell lines, cells were incubated in culture medium containing various concentrations (0-1000 IU/ml) of IFN-ß alone or IFN-ß with 10 μM TMZ for 72 h. As shown in Fig. 5, the combination of IFN-ß and TMZ did not reveal an additive cell growth inhibitory effect in the AM-38, U-87MG and U-251MG cell lines, whereas it did in the A-172, T98G and U-138MG cell lines (the T98G and U-138MG cell lines were resistant to TMZ). The cell growth inhibitory effect of 10 μM TMZ was enhanced by IFN-ß in the TMZ-resistant glioma cell lines T98G and U-138MG.

The T98G cell line, representative of *MGMT* mRNA expression and resistant to TMZ, was chosen to investigate further the synergistic effect of a combination of IFN-ß and TMZ. As shown in Fig. 6, a synergistic cell growth inhibitory effect of combined treatment with IFN-ß and TMZ was significantly observed in the T98G cells. Exposure of T98G cells to 10 IU/ml IFN-ß, 10 μM TMZ, or a combination of

Table I. Comparisons among *MGMT* methylation status, absolute values of *MGMT* mRNA and expression of MGMT protein in six malignant glioma cell lines.

Cell line	Methylation-specific PCR	Absolute value of *MGMT* mRNA (copy/μg RNA)	Western blots of MGMT protein
A-172	Methylated	Not detected	Not detected
AM-38	Methylated	Not detected	Not detected
T98G	Methylated and unmethylated	$5.9x10^3$	Expression
U-87MG	Methylated	Not detected	Not detected
U-138MG	Unmethylated	$6.3x10^3$	Expression
U-251MG	Methylated and unmethylated	Not detected	Not detected

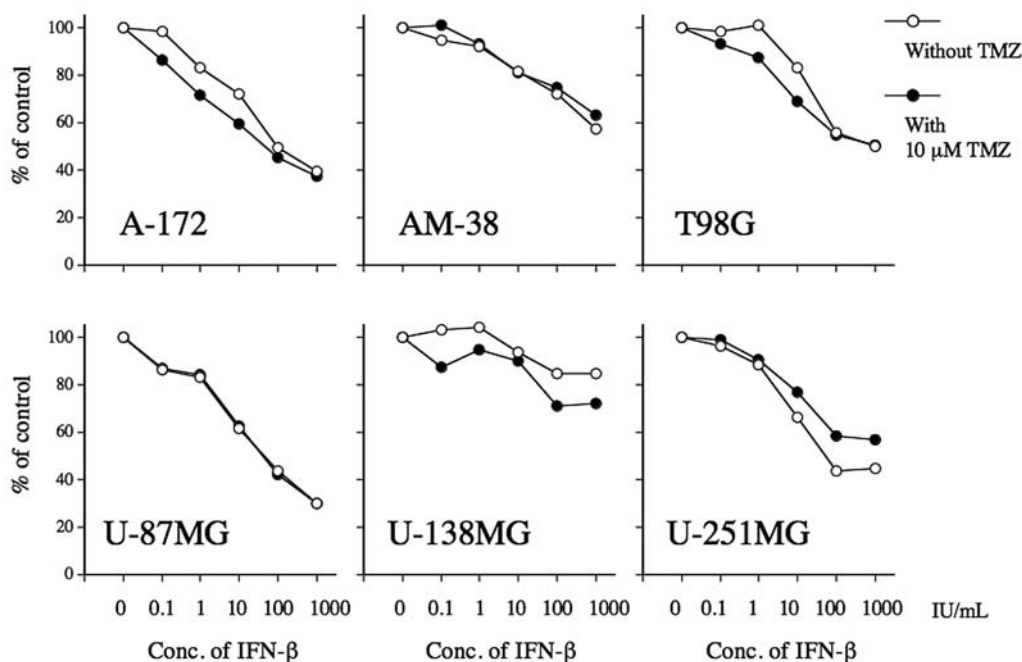The absolute values of *MGMT* mRNA ware normalized to the level of *GAPDH* mRNA.

Figure 5. Cell growth inhibitory effect of a combination of TMZ and IFN-ß in six glioma cell lines. Six malignant glioma-derived cell lines were exposed to medium containing various concentrations of IFN-ß without 10 μM TMZ (○) or with 10 μM TMZ (●) for 72 h. The combination of TMZ and IFN-ß did not reveal an additive cell growth inhibitory effect in the AM-38, U-87MG and U-251MG cell lines, whereas it did so in the A-172, T98G and U-138MG cell lines (the T98G and U-138MG cell lines were resistant to TMZ). The cell growth inhibitory effect of 10 μM TMZ was enhanced by IFN-ß in the TMZ-resistant glioma cell lines, T98G and U-138MG.
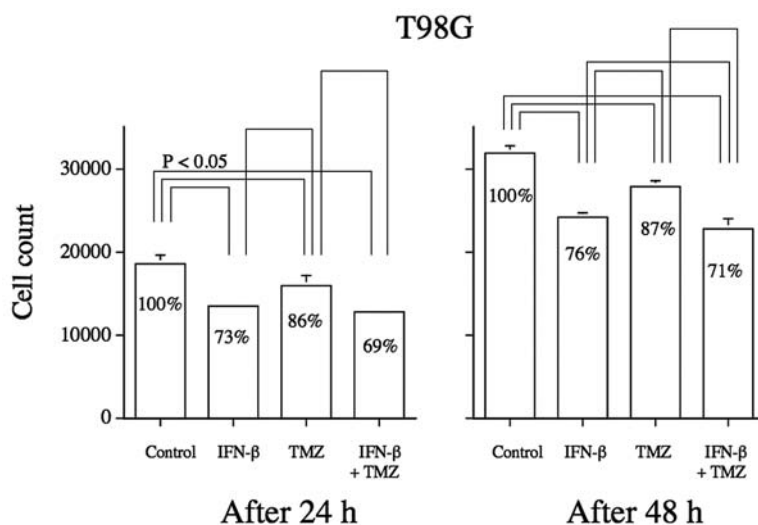

Figure 6. Cell growth inhibitory effect of a combination of IFN-ß and TMZ in TMZ-resistant cells. A synergistic cell growth inhibitory effect was significantly observed in the TMZ-resistant glioma cell line T98G. ANOVA demonstrated a significant difference between the groups after 24 and 48 h, respectively (P<0.0001, respectively), and the Tukey-Kramer test was used to identify which group differences accounted for the significant P-value, respectively (P<0.05).

10 IU/ml IFN-ß and 10 μM TMZ resulted in 73, 86 and 69% of cell growth as compared to the control after 24 h, and 76, 87 and 71% of cell growth as compared to the control after 48 h, respectively. (ANOVA was followed by the Tukey-Kramer test for significant difference assessment among multiple comparisons).

The addition of 10 IU/ml IFN-ß to 10 μM TMZ also reduced the *MGMT* mRNA expression to a level significantly lower than that attained with either agent alone after 6 h in

T98G cells (Fig. 7); the control, 10 IU/ml IFN-ß, 10 μM TMZ, or the combination of 10 IU/ml IFN-ß and 10 μM TMZ resulted in 6.75±0.93, 5.30±0.42, 5.26±1.08 and 4.60±0.52x10^3 copy/μg RNA, respectively. (Parametric comparisons employed ANOVA, and the significance of individual differences was evaluated by using the Tukey-Kramer test). The quantitative *MGMT* mRNA level after exposure to the combination of IFN-ß and TMZ was significantly lower than that in the control [ANOVA revealed
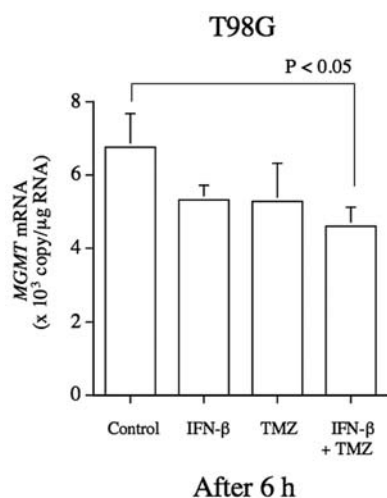
Figure 7. Effect of a combination of IFN-ß and TMZ on the quantitative level of *MGMT* mRNA expression in TMZ-resistant cells. T98G cells were exposed to 10 IU/ml IFN-ß, 10 μM TMZ, or both 10 IU/ml IFN-ß and 10 μM TMZ for 6 h. The addition of 10 IU/ml IFN-ß to 10 μM TMZ reduced the *MGMT* mRNA expression to a level significantly lower than that attained with either agent alone. The *MGMT* mRNA values are expressed as the means ± standard deviation (SD). Experiments were performed at least five times independently.

Figure 8. Induction of IFN-ß protein by TMZ in TMZ-resistant cells. T98G cells were treated with or without 10 μM TMZ for 24, 48 or 72 h, and the cell culture supernatants were assayed for IFN-ß protein by ELISA. No IFN-ß was detectable in the culture supernatants without TMZ. However, detectable amounts of IFN-ß were present in the culture supernatants treated with 10 μM TMZ. The results are expressed as the means (± SD) of six independent measurements.

a significant difference between the groups (P=0.0073), and the Tukey-Kramer test was used to identify which group differences yielded a significant P-value (P<0.05)]. Similar results of a reduced *MGMT* mRNA expression level were obtained upon comparison of the control, 100 IU/ml IFN-ß, 100 μM TMZ and combination of 100 IU/ml IFN-ß with 100 μM TMZ after a 6-h exposure in T98G cells (data not shown).

Significant potentiations of TMZ antitumor activity and *MGMT* mRNA depletion were achieved by the combination with IFN-ß in the TMZ-resistant malignant glioma cell line T98G. Similar inhibition patterns were also observed for U-138MG cells (data not shown).

Endogenous IFN-ß protein induced by TMZ in T98G cells. To determine whether or not TMZ can induce T98G cells to produce IFN-ß protein, cells were treated with or without 10 μM TMZ, and the protein concentrations of the culture supernatants were measured by ELISA at 24, 48 or 72 h after treatment. There were no detectable levels of IFN-ß protein in the supernatants without 10 μM TMZ treatment at 24, 48 or 72 h. In contrast, significant amounts of IFN-ß protein were detected in 10 μM TMZ-treated T98G cells. As shown in Fig. 8, after 24, 48 and 72 h of incubation with the medium containing 10 μM TMZ, the supernatants of the T98G cells contained 11.5±5.1 IU/ml, 15.5±8.7 IU/ml and 18±7.0 IU/ml of IFN-ß protein, respectively. This is the first time that TMZ has been shown to induce endogenous IFN-ß in T98G cells. Several other cell lines employed in this study were also observed to demonstrate IFN-ß protein induction by TMZ treatment (data not shown).

Discussion

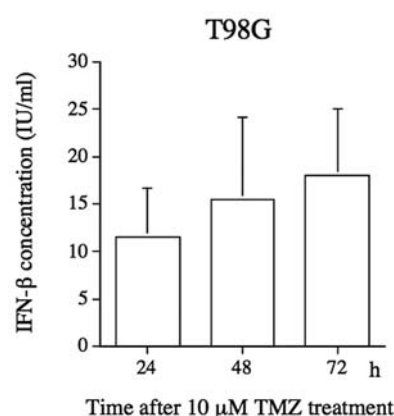Despite optimal treatment involving cytoreductive surgery and radiation therapy, prognosis for malignant gliomas remains poor. As a result, numerous attempts have been made to achieve improved results through chemotherapy. Chloro-ethylating agents (e.g. CCNU and BCNU) and methylating agents (e.g. procarbazine and TMZ) in single-agent or combination chemotherapy, are the principal drugs used in the treatment of malignant gliomas. In recent years, TMZ, with it oral bioavailability, penetration of the blood-brain barrier and safety profile, has emerged as a key agent in the first-line treatment or relapse of malignant gliomas. Although the cytotoxicity of chloroethylating agents and methylating agents can be attributed to quite different DNA lesions, both depend upon initial adduct formation at the O^6-position of guanine (9,55,56). Several preclinical and clinical studies have strongly suggested that the DNA repair enzyme MGMT, with reverse alkylation at the O^6-position of guanine, plays an important role in cellular resistance to chlorethylation or methylation damage at the O^6-position of guanine DNA (10,13-18,26,57-62). In human cancer, the *MGMT* gene is not commonly mutated or deleted, and loss of MGMT function is most frequently due to epigenetic changes, particularly promoter hypermethylation (22,28,63), which cause *MGMT* transcriptional silencing in cells with defective O^6-methyl-guanine repair (22,28,64,65). In the present study, we demonstrated that human malignant glioma-derived cell lines differed in their sensitivity to TMZ, and the MGMT status, including promoter hypermethylation, mRNA expression and protein expression, was strongly correlated with TMZ sensitivity *in vitro*. MGMT may be responsible, at least in part, for the limited benefit afforded by TMZ in the treatment of malignant gliomas. This is consistent with the findings of recently reported clinical studies including our own work that demonstrated that the presence of *MGMT* hypermethylation is associated with a good clinical response and better survival in patients with malignant gliomas who are treated with nitrosourea or TMZ in conjunction with radiation therapy (14,20,21,23-25,27-29).

Although some fractions of gliomas may have a heightened sensitivity to alkylating agents as a consequence of lacking

MGMT activity (21,62), there is an obvious need for newer and more effective therapeutic strategies since the majority of gliomas do express MGMT activity (17,62). Since cellular MGMT activity can be restored only by *de novo* protein synthesis, its depletion, such as with the substrate analogue inhibitor O^6-benzylguanine (O^6-BG), has been proposed as a useful adjuvant to methylating or chloroethylating treatment (56,58,66,67). Further investigations are clearly needed to identify such additional agents with increased efficacy and low toxicity. If MGMT depletion can be accomplished by a drug that also has an antitumor activity, combination therapy with this drug and TMZ could produce a synergistic effect. One candidate is IFN-ß. Its antitumor activities including the contribution of MGMT in TMZ-resistant malignant glioma cells were investigated in the present study, and we demonstrated that a combination of IFN-ß and TMZ treatment synergistically produced inhibitory effects on cell growth and down-regulated *MGMT* mRNA levels in the TMZ-resistant glioma cell line T98G. There was a significantly enhanced antitumor effect compared to that achieved when either agent was used alone.

It has been shown that the concentration of TMZ in the central nervous system is approximately 30 to 40% of the plasma concentration (68). Furthermore, Ostermann *et al* (51) found that, in plasma and cerebrospinal fluid (CSF) concentration-time profiles of TMZ in malignant glioma patients, the TMZ concentrations ranged from 0.10 to 13.39 μg/ml in the plasma and from 0.16 to 1.93 μg/ml (corresponding to ~10 μM) in the CSF. On the other hand, Higuchi and Hashida (52) reported that the plasma concentration levels of IFN-ß were 40 and 96 IU/ml after intravenous administration of 3×10^6 and 6×10^6 IU IFN-ß, respectively, when administered in 60 min. More importantly, we were therefore able to demonstrate that these synergistic effects were observed at clinically relevant concentrations of IFN-ß and TMZ in the CSF, which did not individually induce significant depletion of *MGMT* mRNA when the IFN-ß or TMZ treatment was administered alone. Furthermore, based on the favorable toxicity profile observed in a recent phase II study in which IFN-ß was administered after conventional radiation therapy, it was suggested that IFN-ß represents a particularly good candidate for use in combination with current TMZ-based chemoradiation regimens (69).

Finally, there are two different approaches to the use of IFN in clinical medicine: exogenous IFN (application of purified IFN) and endogenous IFN (induction in the organism of IFN synthesis by exogenous IFN inducers) (39). Although the interest in carrying out investigations on IFN inducers has diminished, endogenous IFN naturally produced by the organism has been found to have the following advantages over exogenous IFN in clinical trials: endogenous IFN is not antigenic, its release can be regulated to prevent oversaturation with IFN, a single injection of IFN inducer can stimulate a relatively prolonged circulation of therapeutic amounts of IFN, and treatment with IFN inducers does not rule out combined therapy with exogenous IFN (39). Furthermore, an orally active IFN inducer could be clinically advantageous in terms of its convenience for long duration administration. At present, all known IFN inducers, e.g. viruses, double-stranded RNA such as polyribonosonic-ribocytidylic acid (poly rI:poly rC), LPS, CpG DNA, pyrimidinones and imidazoquinolinamines, can be divided into two main categories: natural and synthetic. Both groups of inducers can be further divided into low-molecular-weight and high-molecular-weight preparations (39). In the present study, the clinically achievable concentration of 10 μM TMZ, an oral bioavailable alkylating agent of low molecular weight (194 Da), displayed an ability to induce endogenous IFN-ß in T98G cells, at least in certain glioma cells. Marked amounts of IFN-ß protein were detected in T98G cells treated with TMZ, but not in untreated cells. The mechanism responsible for the production of endogenous IFN-ß remains unclear at present. Induction of endogenous IFN-ß production does, however, seem to play an important role in the observed antitumor potency of TMZ, since recent data suggest that the endogenous IFN-ß protein produced by tumor cells inhibits cell growth much more strongly than does added exogenous IFN-ß protein in malignant gliomas (40-46).

The findings presented here suggest that the clinical therapeutic efficacy of TMZ might be enhanced by combination with IFN-ß in malignant gliomas unmethylated at the *MGMT* gene. They also provide an experimental basis for future strategies in TMZ chemotherapy, although further studies are needed to determine the detailed role of combined IFN-ß and TMZ chemotherapy in increasing tumor sensitivity to TMZ chemotherapy and the toxicity profile of combined IFN-ß and TMZ chemotherapy.

Acknowledgements

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