Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor (TNF) family, induces apoptosis in cancer cells by binding to its receptors, death receptor 4 (DR4) and DR5, without affecting normal cells, and is therefore considered to be a promising antitumor agent for use in cancer treatment. However, several studies have indicated that most glioma cell lines display resistance to TRAIL-induced apoptosis. To overcome such resistance and to improve the efficacy of TRAIL-based therapies, identification of ideal agents for combinational treatment is important for achieving rational clinical treatment in glioblastoma patients. The main aim of this study was to investigate whether interferon-β (IFN-β) (with its pleiotropic antitumor activities) could sensitize malignant glioma cells to TRAIL-induced apoptosis using glioma cell lines. TRAIL exhibited a dose-dependent antitumor effect in all of the 7 types of malignant glioma cell lines, although the intensity of the effect varied among the cell lines. In addition, combined treatment with TRAIL (low clinical dose: 1 ng/ml) and IFN-β (clinically relevant concentration: 10 IU/ml) in A-172, AM-38, T98G, U-138MG and U-251MG demonstrated a more marked antitumor effect than TRAIL alone. Furthermore, the antitumor effect of the combined treatment with TRAIL and IFN-β may be enhanced via an extrinsic apoptotic system, and upregulation of DR5 was revealed to play an important role in this process in U-138MG cells. These findings provide an experimental basis to suggest that combined treatment with TRAIL and IFN-β may offer a new therapeutic strategy for malignant gliomas.

Introduction

Malignant gliomas, especially glioblastomas, are the most frequent and devastating primary tumors of the central nervous system (1,2). Despite improvements in multimodality treatments consisting of combinations of surgical resection, irradiation, and chemotherapy, the prognosis for glioblastoma patients remains dismal (3,4). Novel treatment strategies for patients with glioblastomas are thus urgently required.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor (TNF) family, and induces apoptosis in cancer cells by binding to its receptors, death receptor 4 (DR4) and DR5 (5-9). Although DR4 and DR5 are highly expressed in cancer cells, such expression is limited in normal cells (10,11). TRAIL has thus been expected to represent one of the most promising agents for use in cancer treatment (5-8). In a study employing malignant glioma specimens, it was demonstrated that the expression of DR4 was 75% and that of DR5 was 95% (12). However, several investigations have indicated that most glioma cell lines display resistance to TRAIL-induced apoptosis (12,13). Recently, various combination treatments with other drugs as a sensitizer have been attempted and have yielded promising results (14-18). To overcome the resistance to TRAIL-induced apoptosis and to improve the efficacy of TRAIL-based therapies, identification of ideal agents for combination treatment is important for achieving rational clinical treatment in glioblastoma patients.

Human interferon-β (IFN-β) is a cytokine which belongs to the type I IFNs (19). It was originally identified as an antiviral agent, and has been revealed to exhibit pleiotropic antitumor activities including an anti-angiogenic activity, immunomodulatory activity, growth inhibition, and apoptosis induction (20,21). In addition to such multiple functions of IFN-β against human neoplasias, it can act as a drug sensitizers.
sensitizer enhancing the antitumor effect when administered in combination with other agents in the treatment of malignant gliomas (22-26). IFN-β binds to the cell membrane receptor, interferon-α/β receptor (IFNAR), and acts by enhancing the expression of IFN-stimulated genes (ISGs) through the signal induction of the Janus kinase (JAK)/signal transducer and activator of the transcription (STAT) signaling pathway. To date, over 300 ISGs have been identified, including genes such as TRAIL and Fas, suggesting a strong involvement with exogenous apoptosis. Furthermore, several recent studies have demonstrated that the apoptotic activity of IFN depends partly on the TRAIL signaling pathway (27,28).

The aforementioned findings suggest that favorable therapeudic interactions could occur between TRAIL and IFN-β. Therefore, in the present study, the potential sensitizing effects of IFN-β towards TRAIL-induced apoptosis was investigated in malignant glioma cells aiming to provide an experimental basis for rational clinical treatments in glioblastoma patients.

Materials and methods

Cell lines, culture conditions and materials. Human malignant glioma cell lines A-172 (cell no. JCRB0228; lot no. 021999), AM-38 (cell no. IFO50492; lot no. 12082003), T98G (cell no. IFO50303; lot no. 1007), U-251MG (cell no. IFO50288; lot no. 12132002), and YH-13 (cell no. IFO50493; lot no. 1164) were purchased from Health Science Research Resources Bank (Sennan, Osaka, Japan). U-87MG (glioblastoma of unknown origin; cat. no. HTB-14; lot no. 2497162) and U-138MG (cat. no. HTB-16; lot no. 1104428) were purchased from the American Type Culture Collection (Manassas, VA, USA). In a previous study, we confirmed that O6-methylguanine-DNA methyltransferase (MGMT, a key factor of alkylating agents) is expressed in T98G, U-138MG and YH-13 cells by real-time RT-PCR and western blot analysis (29). Consistent with an earlier study (30), it was also confirmed that T98G (237 Met→Ile) and U-251MG (273 Arg→His) have a point mutation in the p53 gene (data not shown).

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co., Ltd.) supplemented with 10% fetal calf serum (FCS; Life Technologies; Thermo Fisher Scientific, Inc.) using plastic culture flasks (Corning, Inc.) in a 37°C humidified incubator with 5% CO2. Natural-type IFN-β (Toray Industries, Inc.) and TRAIL (Wako Pure Chemical Industries, Ltd.) were employed for the experiments.

Cell viability analysis. Cells were seeded at 1x10^6 cells/well in 24-well plates. After 24 h of attachment, the cells were further incubated with fresh medium containing TRAIL, and/or IFN-β for 72 h. To determine the cell viability, the surviving cells in each well were counted using a Coulter Counter (Coulter Counter Z1; Beckman Coulter, Inc.) after confirming the presence of living cells with 0.45% trypan blue solution (Sigma-Aldrich; Merck KGaA). The experiments were repeated 6 times at each concentration.

Further treatment conditions were set with TRAIL at 1 ng/ml and IFN-β at 10 IU/ml, since the cell growth inhibitory effect was significant when TRAIL was at 1 ng/ml or more, and 10 IU/ml of IFN-β represents a clinically relevant concentration (26,31). In phase II RCS for non-small cell carcinoma and B cell lymphoma, 8 mg/kg of TRAIL was administered, and its blood concentration reached about 80 µg/ml (32,33). The dose of TRAIL (1 ng/ml) employed in the following experiments was thus considered to be a low clinical dose.

Since U-138MG displayed a marked antitumor effect at a small amount (0.1 and 1 ng/ml) of IFN-β, these cells were employed in the following experiments.

Analysis of apoptosis by flow cytometry. Cells were seeded at 1x10^6 cells/well in 6-well plates (Corning, Inc.) and cultured for 24 h. Subsequently, the cells were further incubated with fresh medium (control), medium containing TRAIL (1 ng/ml) and/or IFN-β (10 IU/ml) for 72 h. The cells were washed with phosphate-buffered saline (PBS) and collected using trypsin-EDTA solution. After suspension with 100 µl binding buffer, 5 µl of Annexin V Alexa Fluor 488 conjugate (Invitrogen; Thermo Fisher Scientific, Inc.) and 10 µl of propidium iodide solution (PI; Miltenyi Biotec, Inc.) were added, and the cells were incubated at room temperature for 15 min. Stained cells were analyzed with a fluorescence-activated cell sorter (FACS)-Calibur flow cytometer (BD Biosciences). The experiments were repeated 3 times to confirm reproducibility.

Western blot analysis. Proteins were isolated from 1x10^7 cells using RIPA buffer (Wako Pure Chemical Industries, Ltd.) supplemented with protease inhibitor complex mix (Roche Diagnostics). The protein concentrations were determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc.). A total of 50 µg of protein was separated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (TEFCO, Inc.) and transferred onto nitrocellulose membranes (GE Healthcare) for 30 min at 15 V employing Bio-Rad Trans Blot (Bio -Rad Laboratories, Inc.). The membranes were blocked with 1% skimmed milk dissolved in washing buffer (PBS + 0.1% Tween-20) for 60 min at room temperature. The membranes were incubated with primary antibodies diluted according to the manufacturer's instructions at 4°C overnight (anti-caspase-3 rabbit mAb, cat. no. 9665; 1:1,000 dilution; anti-caspase-8 mouse mAb cat. no. 9746; 1:1,000 dilution; and anti-caspase-9 mouse mAb, cat. no. 9508; 1:1,000 dilution) (Cell Signaling Technology, Inc.). Anti-β-actin mouse mAb (cat. no. 013-24553; 1:2,000 dilution; Wako Pure Chemical Industries, Ltd.) was utilized as a loading control. Anti-mouse or anti-rabbit IgG (cat. no. A4416; 1:5,000 dilution; Sigma-Aldrich; Merck KGaA, cat. no. 7074; 1:5,000 dilution; Cell Signaling Technology, Inc., respectively) was employed as the secondary antibody for 60 min at room temperature. The band patterns were analyzed using ImageQuant LAS-4000 after treatment with ECL Prime Western Blotting Detection Reagent (both from GE Healthcare). The same experiments were repeated 3 times to confirm reproducibility.

Real-time reverse transcription-PCR analysis. RNA extraction was performed using an RNeasy Mini kit (Qiagen, Inc.). Relative mRNA expression was evaluated by real-time reverse transcription-PCR (qRT-PCR) employing a StepOne Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and a One-Step qPCR kit (SYBR® Green Real-time
PCR Master Mix (Toyobo Life Science) according to the manufacturers’ instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was utilized as an internal control.

The following primers were used in this experiment (17,34-36): GAPDH forward, 5'-CAG AAC ATC ATC CCT GCC TCT-3' and reverse, 5'-GCT TGA CAA AGT GGT CGT TGA G-3'; DR4 forward, 5'-TGT ACG CCT GGA GTG ACA T-3' and reverse, 5'-CACCAACAGCAACGGAACAA-3'; DR5 forward, 5'-CAG GTG TCA ACATGTGGTCC-3' and reverse, 5'-ATCGAA GCAGTGCTGAGAG-3'; cellular FLICE inhibitory protein (c-FLIP) forward, 5'-GGAGCTATAGAGTGTGATGG-3' and reverse, 5'-GGCCACTTCCACTGACTAA-3'; B-cell lymphoma 2-associated x protein (Bax) forward, 5'-TTTGCTTGAAGGGTCTCAT CC-3' and reverse, 5'-CAGTTGAAGTTGCCGTCAGA-3'. The thermocycling conditions were 90˚C for 30 sec, 61˚C for 20 min, and 95˚C for 1 min, followed by 40 cycles at 95˚C for 15 sec, 55˚C for 15 sec and 74˚C for 45 sec. The expression levels were calculated employing the following equations by comparing the threshold cycle (CT): ∆Cq = CT of DR4, DR5, FAS, p53, Bax, or c-FLIP-CT of GAPDH, ∆∆Cq (target cell line)-∆Cq (reference cell line), and ratio = 2-∆∆Cq (37). The experiments were repeated 3 times under each condition.

Influence of DR5 blocking antibody on the antitumor effect of combined treatment with TRAIL and IFN-β. In order to evaluate the involvement of DR5 in the antitumor effect of TRAIL in combination with IFN-β, the anti-apoptotic effect of administering DR5 blocking antibody was examined. U-138MG cells were seeded at 5x10⁴ cells/well in 24-well plates. After 24 h of attachment, the cells were further incubated with fresh medium containing 10 ng/ml DR5 blocking antibody (Recombinant Human TRAIL R2/TNFRSF10B Fc Chimera Protein; R&D Systems, Inc.), or 1 ng/ml TRAIL and 10 IU/ml IFN-β without/with DR5 blocking antibody (2.5, 5 and 10 ng/ml) for 72 h. The surviving cells in each well were then counted using a Coulter Counter (Coulter Counter Z1; Beckman Coulter, Inc.) after confirming the presence of living cells with 0.45% trypan blue solution (Sigma-Aldrich; Merck KGaA). The experiments were repeated 6 times at each concentration.

Figure 1. Antitumor effects of TRAIL (0-1,000 ng/ml) or IFN-β (0-1,000 IU/ml) against 7 types of malignant glioma cell lines (A-172, AM-38, T98G, U-87MG, U-138MG, U-251MG and YH-13). Both TRAIL (left) and IFN-β (right) exhibited a cell growth inhibitory effect in a dose-dependent manner in all cell lines at 72 h. Data are expressed as a percentage of the control. Compared to the no treatment group and the treatment groups, *P<0.05. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IFN-β, interferon-β.

Statistical analysis. The experiments were repeated at least 3 times under each condition. Where the same experiment was performed more than 6 times, the mean value and standard error (SE) were calculated and utilized. Mann-Whitney’s U test was carried out to compare data between pairs of groups. The Tukey-Kramer test was conducted to perform comparisons of three or more groups, and when there was a significant
difference, Student’s t-test was employed as a subsequent test. For the data analyses, the statistical software SPSS (version 21.0: IBM Corp.) was used.

Results

Antitumor effect of TRAIL and IFN-β in malignant glioma cells. To evaluate the antitumor effect of TRAIL and IFN-β, the 7 malignant glioma cell lines (A-172, AM-38, T98G, U-87MG, U-138MG, U-251MG and YH-13) were treated with 0-1,000 ng/ml of TRAIL or 0 -1,000 IU/ml of IFN-β for 72 h, and then assessed by counting the viable cells in the media. In all cell lines, both TRAIL and IFN-β caused a decrease in cell viability in a dose-dependent manner as revealed in Fig. 1.

Antitumor effect of a combination of TRAIL and IFN-β. To assess whether or not combined treatment with TRAIL and IFN-β could exert a synergistic effect in the 7 malignant glioma cell lines, cells were treated with various concentrations of TRAIL (0-1,000 ng/ml) or TRAIL with 10 IU/ml of IFN-β for 72 h, and then assessed by counting the viable cells in the media. In all cell lines, both TRAIL and IFN-β caused a decrease in cell viability in a dose-dependent manner as revealed in Fig. 1.

These results did not appear to be related to the MGMT or the p53 status in the case of TRAIL or TRAIL and IFN-β sensitivity. Because the additive cell growth inhibitory effect of combined treatment with TRAIL and IFN-β was observed with T98G and U-138MG, but not with YH-13, although these three cell lines are MGMT positive. In addition, the additive cell growth inhibitory effect of combined treatment with TRAIL and IFN-β was observed with the A-172, AM-38, T98G, U-138MG and U-251MG cell lines, although A-172, AM-38, U-87MG, U-138MG and YH-13 are wild-types of p53 status, in contrast T98G and U-251MG are mutant types of p53 status.

Detection of apoptosis by flow cytometry and western blotting. To assess the apoptosis induced by TRAIL and IFN-β, flow cytometry with Annexin V/PI double staining was performed (Annexin V-positive, early-stage apoptosis; Annexin V/PI-positive, late-stage apoptosis). As revealed in Fig. 3, combined treatment with TRAIL (1 ng/ml) and IFN-β (10 IU/ml) induced significant apoptosis after 72 h in U-138MG cells. The proportion of Annexin V/PI positive cells following combined treatment with TRAIL and IFN-β (mean = 7.99%) was higher than that for each single agent (means: Untreated, 2.71%; TRAIL, 3.77%; and IFN-β, 4.13%, respectively).

Furthermore, to evaluate the underlying mechanisms of the apoptotic effect of TRAIL and IFN-β, the protein expression of caspase-3 (an effector caspase), caspase-8 (extrinsic apoptotic pathway), and caspase-9 (intrinsic mitochondrial
pathway) were evaluated by western blot analysis. As revealed in Fig. 4, following 24 h of combined treatment with TRAIL and IFN-β, the protein expression levels of cleaved caspase-8 and cleaved caspase-3 were revealed to be more marked when compared to those following treatment with TRAIL or IFN-β alone. However, as regards the protein expression of caspase-9 and cleaved caspase-9, no difference was noted among the treatments. The effect of combined treatment with TRAIL and IFN-β in U-138MG may thus be due to promotion of apoptosis through the exogenous apoptotic pathway.

Expression of apoptosis-related genes. To further elucidate the role of IFN-β in combined treatment with TRAIL and IFN-β, the mRNA expression levels of apoptosis-related genes were evaluated by real-time qRT-PCR. The mRNA expression levels of DR4, DR5, Fas, p53, Bax and c-FLIP were determined following 4 h of IFN-β treatment in U-138MG cells. Significant upregulation of DR5 (mean: 2.97±0.23-fold), Fas (mean: 1.65±0.11-fold), and p53 (mean: 1.50±0.11-fold) was detected; however, no significant changes in DR4 (mean: 1.07±0.01-fold), Bax (mean: 1.17±0.11-fold), and c-FLIP (mean: 0.89±0.23-fold) were revealed (Fig. 5).

Apoptosis inhibition by DR5 blocking antibody. To confirm that the antitumor effect of combined treatment with TRAIL and IFN-β in malignant glioma cells is dependent on DR5, the number of surviving cells was counted after treatment for 72 h with DR5 blocking antibody (2.5, 5 or 10 ng/ml). The DR5 blocking antibody alone displayed no significant effect on U-138MG cells, and combined treatment with TRAIL (1 ng/ml) and IFN-β (10 IU/ml) exhibited a significant cell growth inhibitory effect (mean: 39.3±10.4% when compared to no treatment). The DR5 blocking antibody caused a significant attenuation of the inhibitory effect on cell proliferation.
glioma cell lines demonstrated a more significant antitumor effect than TRAIL alone. To the best of our knowledge, this is the first time that IFN-β enhancement of TRAIL-induced apoptosis has been observed in particular malignant glioma cells. In the future, one important issue requiring examination will be the antitumor effect occurring after application of TRAIL and IFN-β in combination with TMZ, which is the current standard therapeutic agent for malignant gliomas. In addition, once sufficient data have been gathered, the morphological changes of the cells associated with TRAIL, IFN-β, and combined treatment will require investigation.

Higuchi and Hashida (31) reported that the plasma concentration levels of IFN-β were 40 and 96 IU/ml following intravenous administration of $3 \times 10^5$ and $6 \times 10^5$ IU IFN-β, respectively, when administered in 60 min. On this basis, 10 IU/ml is considered a clinically relevant concentration of IFN-β. Furthermore, in phase II RCS for non-small cell lung cancer and B cell lymphoma, 8 mg/kg of TRAIL was administered, and its plasma concentration reached ~80 mg/ml (32,33). The concentration of TRAIL used in the present study is therefore considered to be clinically low. In particular, in A-172, AM-38, T98G, and U-138MG cells, a cell growth inhibitory effect was also significantly observed even at a small amount (0.1 and 1 ng/ml) of TRAIL with 10 IU/ml of IFN-β, suggesting that IFN-β represents a good candidate for use in combination with TRAIL-based treatment regimens for malignant gliomas. However, based on the present study, we do not have sufficient data to elucidate the exact differences in sensitivity to each drug between each cell line. Furthermore, the detailed mechanisms underlying such differences remain unidentified in literature, and this presents our next topic of research. In the present study, the focus was on evaluating in more detail how the combined treatment with TRAIL and IFN-β can provide a more marked antitumor effect.

Following combined treatment with TRAIL (1 ng/ml) and IFN-β (10 IU/ml), FACS analysis indicated that late-phase apoptosis or necrosis was increased (42), when compared to that with TRAIL or IFN-β alone in U-138MG cells. Furthermore, it was revealed by western blotting that caspase-8 and caspase-3, which are involved in the extrinsic apoptotic pathways (17,34-46), were cleaved by combined treatment with TRAIL and IFN-β, whereas caspase-9 which is involved in the intrinsic apoptotic pathway exhibited no cleavage. The antitumor effect of combined treatment with TRAIL and IFN-β may thus be enhanced via an extrinsic apoptotic system in U-138MG cells.

TRAIL induces apoptosis of cancer cells by binding to its receptors DR4 and DR5 (9). In malignant glioma cells, there have been indications that cisplatin and irradiation may upregulate the expression of DR5 (38,47). Conversely, in malignant glioma cells, IFN-β has been revealed to upregulate the expression of p53 (24). Furthermore, it has been suggested that p53 promotes the expression of DR5 (48). In the present study, it was demonstrated and confirmed that the quantitative p53 and DR5 mRNA levels were upregulated by IFN-β (10 IU/ml), however, no significant upregulation was observed for DR4 in U-138MG cells. In the future, it is surmised that it will also be necessary to investigate the protein levels produced by such apoptosis-related genes following IFN-β treatment. In addition, as revealed in Fig. 6, the antitumor effect elicited by the combined treatment with TRAIL and IFN-β was significantly
attenuated depending on the concentration of DR5 blocking antibody. These findings indicated that upregulation of DR5 via p53 by IFN-β may play an important role in the enhanced antitumor effect of combined treatment with TRAIL and IFN-β in U-138MG, although the investigation of the effect of DR5 blocking antibody with TRAIL or INF-β treatment alone is required. Moreover, Fas, which is a receptor that induces apoptosis (extrinsic apoptotic pathway) (46), was also associated with increased expression levels of quantitative mRNA by IFN-β in the present study. These circumstances were considered to represent part of the mechanism whereby the antitumor effect was enhanced by employing IFN-β in combination with TRAIL.

In conclusion, in the present study, it was demonstrated that combined treatment with a clinically relevant concentration of TRAIL and IFN-β produced a significantly enhanced antitumor effect in malignant glioma cells as compared to that achieved when either agent was used alone, and this may be partially dependent on DR5 through the extrinsic pathway of apoptosis. Although the present findings were not sufficient

Figure 5. Apoptosis-related mRNA expression levels of DR4, DR5, Fas, p53, Bax, and c-FLIP following IFN-β treatment as analyzed by real-time qRT-PCR. Significant upregulation of DR5, Fas, and p53 was observed in the case of IFN-β (10 IU/ml) treatment for 4 h in U-138MG cells. However, there were no differences in DR4, Bax, and c-FLIP following 4 h of IFN-β treatment in U-138MG cells. Data are presented as the means ± SE (standard error). *P<0.05 (Student’s t-test). DR4, death receptor 4; DR5, death receptor 5; c-FLIP, cellular FLICE inhibitory protein; Bax, B-cell lymphoma 2-associated x protein; IFN-β, interferon-β.

Figure 6. Cell growth after treatment with DR5 blocking antibody. The DR5 blocking antibody (10 ng/ml) alone displayed no significant effect on U-138MG cells after 72 h. The antibody significantly attenuated the antitumor effect of combined treatment with TRAIL and IFN-β in U-138MG, although the investigation of the effect of DR5 blocking antibody with TRAIL or INF-β treatment alone is required. Moreover, Fas, which is a receptor that induces apoptosis (extrinsic apoptotic pathway) (46), was also associated with increased expression levels of quantitative mRNA by IFN-β in the present study. These circumstances were considered to represent part of the mechanism whereby the antitumor effect was enhanced by employing IFN-β in combination with TRAIL.

Finally, c-FLIP is expressed in tumor cells to a certain level, and competes with caspase-8 to inhibit apoptosis by binding to Fas-associated death domain (49,50). It has been reported that various drugs lower the expression of c-FLIP, and the induction of apoptosis by TRAIL could be enhanced (49-53). It was therefore investigated whether the expression of c-FLIP was decreased by the administration of IFN-β. However, in the present study, no significant decrease in quantitative c-FLIP mRNA level by IFN-β was observed in U-138MG cells.

In conclusion, in the present study, it was demonstrated that combined treatment with a clinically relevant concentration of TRAIL and IFN-β produced a significantly enhanced antitumor effect in malignant glioma cells as compared to that achieved when either agent was used alone, and this may be partially dependent on DR5 through the extrinsic pathway of apoptosis. Although the present findings were not sufficient
to yield a conclusive theory of the mechanism of action, and in vivo experiments will also need to be undertaken in the future, the data did provide an experimental basis to suggest that combined treatment with TRAIL and IFN-β could offer a new therapeutic strategy for malignant gliomas.

Acknowledgements

The authors are grateful to Mr. Hiroyuki Satake and Mr. Nobuo Miyazaki, Toray Industries Inc. (Tokyo, Japan) for their invaluable discussions. Some parts of this study have been incorporated within a Japanese-language thesis submitted for Sodai Yoshimura’s Ph.D. degree at Nihon University School of Medicine (Tokyo, Japan).

Funding

The present study was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (grant no. 16K10772) and in part by a grant from the Health Sciences Research Institute, Inc. (Yokohama, Japan) for the Division of Companion Diagnostics, Department of Pathology and Microbiology, Nihon University School of Medicine (Tokyo, Japan).

Availability of data and materials

All data related to this study are included in this article.

Authors’ contributions

SYo and ES developed the experimental design, performed most of the experiments and analysis, and drafted the basic manuscript. YH and SYa were involved in the conception and design of the study, undertook part of the experiments, analyzed the data, and contributed to the writing of the draft manuscript. KS also conducted some experiments and analyzed the data. TU, TN, HH, and YK were also involved in the conception and design of the study and proofread the manuscript. AY contributed to the experimental design and the writing of the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests with regard to the subjects discussed in this study. TU received funds for other research projects not related to this study from the Ministry of Education, Culture, Sports, Science and Technology, Japan, the Ministry of Economy, Trade and Industry, Japan and the Human Frontier Science Program, while AY received research funds for another research project from Medtronic Japan Co., Ltd.

References

1. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, Ohgaki H, Wiestler OD, Kleihues P and Ellison DW: The 2016 World Health Organization Classification of Tumors of the Central Nervous System: A summary. Acta Neuropathol 131: 803-820, 2016.

2. Report of brain tumor registry of Japan (2015-2017). Neuro Med Chir (Tokyo) 59 (Suppl): S1-S81, 2019.

3. Mahler EA, Furnari FB, Bachoo RM, Rowitch DH, Louis DN, Cavenee WK and DePinho RA: Malignant glioma: Genetics and biology of a grave matter. Genes Dev 15: 1311-1333, 2001.

4. Stupp R, Mason WP, van den Bent MJ, Weller M, Fischer B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, et al: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352: 978-996, 2005.

5. Piti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A and Ashkenazi A: Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem 271: 12687-12690, 1996.

6. Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, et al: Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med 5: 157-163, 1999.

7. Ashkenazi A, Holland P and Eckhardt SG: Ligand-based targeting of apoptosis in cancer: The potential of recombinant human apoptosis ligand 2/tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL). J Clin Oncol 26: 3621-3630, 2008.

8. Naoum GE, Buchsbaum DJ, Tawadros F, Farooqi A and Arafat WO: Journey of TRAIL from bench to bedside and its potential role in immuno-oncology. Oncol Rev 11: 332, 2017.

9. von Karstedt S, Montinaro A and Walczak H: Exploring the TRAILs less travelled: TRAIL in cancer biology and therapy. Nat Rev Cancer 17: 352-366, 2017.

10. Suliman A, Lam A, Datta R and Srivastava RK: Intracellular mechanisms of TRAIL: Apoptosis through mitochondrial-dependent and -independent pathways. Oncogene 20: 2122-2133, 2001.

11. Nagane M, Huang HJ and Cavenee WK: The potential of TRAIL for cancer chemotherapy. Apoptosis 6: 191-197, 2001.

12. Kuijlen JM, Bremer E, Mooij JJ, den Dunnen WF and Helfrich W: Review: On TRAIL for malignant glioma therapy? Neuropathol Appl Neurobiol 36: 168-182, 2010.

13. Hawkins CJ: TRAIL and malignant glioma. Vitam Horm 67: 427-452, 2004.

14. Shang Z and Zhang L: Digitoxin increases sensitivity of glioma stem cells to TRAIL-mediated apoptosis. Neurosci Lett 653: 19-24, 2017.

15. Khan M, Bi Y, Qazi JI, Fan L and Gao H: Evodiamine sensitizes U87 glioblastoma cells to TRAIL via the death receptor pathway. Mol Med Rep 11: 257-262, 2015.

16. Badr CE, Wurdinger T, Nilsson J, Niers JM, Whalen M, Degterev A and Tannous BA: Lanatoside C sensitizes glioblastoma cells to tumor necrosis factor-related apoptosis-inducing ligand and induces an alternative cell death pathway. Neuro Oncol 13: 1213-1224, 2011.

17. Yoon MJ, Kang YJ, Kim YK, Kim EH, Lee JA, Lim JH, Kwon TK and Choi KS: Monensin, a polyether ionophore antibiotic, overcomes TRAIL resistance in glioma cells via endoplasmic reticulum stress, DR5 upregulation and c-FLIP downregulation. Carcinogenesis 34: 1918-1928, 2013.

18. Calzolari A, Saule E, De Angelis ML, Pasquini L, Boe A, Pelachic F, Ricci-Vitiani L, Baiocchi M and Testa U: Salinomycin potentiates the cytotoxic effects of TRAIL on glioblastoma cell lines. PLoS One 9: e94438, 2014.

19. Borden EC, Sen GC, Uze G, Silverman RH, Ransohoff RM, Foster GR and Stark GR: Interferons at age 50: Past, current and future impact on biomedicine. Nat Rev Drug Discov 6: 975-990, 2007.

20. Isaacs A and Lindenmann J: Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci 147: 258-267, 1957.

21. Vannucchi S, Chiantore MV, Mangino G, Percario ZA, Affabris E, Fiorucci G and Romeo G: Perspectives in biomolecular therapeutic intervention in cancer: From the early to the new strategies with type I interferons. Curr Med Chem 14: 667-679, 2007.
22. Yoshida J, Kajita Y, Wakabayashi T and Sugita K: Long-term follow-up results of 175 patients with malignant glioma: Importance of radical tumor resection and postoperative adjuvant therapy by concomitant suppression of c-Flip and Mcl-1. Cell Death Differ 20: 875-884, 2013.

23. Watanabe T, Katayama Y, Yoshino A, Komine C, Yokoyama T and Fukushima T: Treatment of low-grade diffuse astrocytomas by surgery and human fibroblast interferon without radiation therapy. J Neurooncol 36: 1367-1377, 2001.

24. Yoshida J, Okagami A, Yachi K, Ohita T, Fukushima T, Watanabe T, Katayama Y, Okamoto Y, Naruse N and Sano E: Effect of IFN-beta on human glioma cell lines with temozolomide resistance. Int J Oncol 35: 139-148, 2009.

25. Der SD, Zhou A, Williams BR and Silverman RH: Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci USA 95: 15623-15628, 1998.

26. Chawla-Sarkar M, Lindner DJ, Liu YF, Williams BR, Sen GC, Yoshino A, Ogino A, Yachi K, Ohta T, Fukushima T, Watanabe T, Katayama Y, Komine C, Yokoyama T, Yoshida J, Kajita Y, Wakabayashi T and Sugita K: Long-term follow-up results of 175 patients with malignant glioma: Importance of radical tumor resection and postoperative adjuvant therapy by concomitant suppression of c-Flip and Mcl-1. Cell Death Differ 20: 875-884, 2013.

27. Yoshino A, Ogino A, Yachi K, Ohita T, Fukushima T, Watanabe T, Katayama Y, Okamoto Y, Naruse N, Sano E and Tsumoto K: Gene expression profiling predicts response to temozolomide in glioblastoma-derived stem cells through up-regulation of DR5 and down-regulation of c-FLIP. Cancer Invest 29: 511-520, 2011.

28. Wu GS, Burns TF, McDonald ER II, Jiang W, Meng R, Krantz ID, Kao G, Gan DD, Zhou JY, Muschel R, et al: KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat Genet 17: 141-143, 1997.

29. Huang Y, Yang X, Xu T, Kong Q, Zhang Y, Shen Y, Wei Y, Wang G and Chang KJ: Overcoming resistance to TRAIL-induced apoptosis in solid tumor cells by simultaneously targeting death receptors, c-FLIP and IAPs. Int J Oncol 49: 153-163, 2016.

30. Min KJ, Um HJ, Seo SU, Woo SM, Kim S, Park JW, Lee HS, Kim SH, Choi YH, Lee TJ and Kwon TK: Angelicin potentiates TRAIL-induced apoptosis in renal carcinoma Caki cells through activation of caspase 3 and down-regulation of c-FLIP expression. Drug Dev Res 79: 3-10, 2018.

31. Lemke J, von Karstedt S, Abd El Hay M, Conti A, Arce F, Montinaro A, Papenfuss K, El-Bahrawy MA and Walczak H: Selective CDK9 inhibition overcomes TRAIL resistance by concomitant suppression of cFlip and Mcl-1. Cell Death Differ 21: 491-502, 2014.

32. Lehn E, Thome M, Mahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schröter M, Burns K, Mattmann C, et al: Inhibition of death receptor signals by cellular FLIP. Nature 388: 190-195, 1997.