どの報告がみられている[69]。BTSCと神経幹細胞や前駆細胞との相同性が多く認められることから，これらの細胞間で同様のシグナル伝達経路が使用されている可能性も高く，神経幹細胞の増殖阻止因子がBTSCの治療薬に発展していくことも予想される[70]。

しかし，BTSCの概念はまだ十分に確立しているとはいえず，どのような幹細胞nicheが必要であるか，その特異的なマーカーの同定や神経膠腫発生における意義や存在部位，耐性・再発への関与と分子機序など，解明されるべき課題は多く残されている。

おわりに

抗癌剤に対する薬剤耐性には薬理学的作用から分子腫瘍学的機序まで多岐にわたる耐性機構が関与していると考えられるが[1]，悪性glioma治療においては血液脳関門の存在や使用される薬剤の種類など，他臓器癌とは異なる検討が必要である。特に本邦も含めTMZが悪性gliomaの標準的治療薬として広く使用されるようになり，MGMTを中心としたTMZ耐性機序の解明とその克服は極めて重要な課題といえる[6]。新規治療法として期待される分子標的治療薬による臨床試験では，当初予想されたような治療効果とバイオマーカーとの相関が必ずしも得られていないのが現状であり，腫瘍細胞内外での複合的シグナル伝達ネットワークの解析と個別化した多重標的治療が試みられている。今後，腫瘍形成・維持・再生にかかわるBTSC仮説の分子機構の解明が，悪性gliomaの治療成績が向上するための次なるbreakthroughとなる可能性もあり，これらの領域での研究発展が切に望まれる。

文 献

1) 永根基雄: 薬剤耐性関連遺伝子と個別化化学療法. 日本臨牀 **63**: 460-471, 2005.
2) Stupp R, Mason WP, van den Bent MJ, *et al*: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* **352**: 987-996, 2005.
3) Frei JV, Swenson DH, Warren W, *et al*: Alkylation of deoxyribonucleic acid in vivo in various organs of C57BL mice by the carcinogens N-methyl-N-nitrosourea, N-ethyl-N-nitrosourea and ethyl methanesulphonate in relation to induction of thymic lymphoma. Some applications of high-pressure liquid chromatography. *Biochem J* **174**: 1031-1044, 1978.
4) Gerson SL: MGMT: its role in cancer aetiology and cancer therapeutics. *Nat Rev Cancer* **4**: 296-307, 2004.
5) Karran P and Bignami M: DNA damage tolerance, mismatch repair and genome instability. *Bioessays* **16**: 833-839, 1994.
6) 廣瀬雄一，佐野公俊: DNAメチル化剤temozolomideの分子薬理学. *No Shinkei Geka* **35**: 117-129, 2007.
7) Gerson SL: Clinical relevance of MGMT in the treatment of cancer. *J Clin Oncol* **20**: 2388-2399, 2002.
8) 永根基雄，口野嘉幸，浅井昭雄: ACNU耐性とMGMTの発現. in *Mebio*, 1993, pp107-113.
9) Schold SC, Jr., Brent TP, von Hofe E, *et al*: O^6-alkylguanine-DNA alkyltransferase and sensitivity to procarbazine in human brain-tumor xenografts. *J Neurosurg* **70**: 573-577, 1989.
10) Friedman HS, Johnson SP, Dong Q, *et al*: Methylator resistance mediated by mismatch repair deficiency in a glioblastoma multiforme xenograft. *Cancer Res* **57**: 2933-2936, 1997.
11) Liu L, Markowitz S, Gerson SL: Mismatch repair mutations override alkyltransferase in conferring resistance to temozolomide but not to 1,3-bis (2-chloroethyl) nitrosourea. *Cancer Res* **56**: 5375-5379, 1996.
12) Esteller M, Garcia-Foncillas J, Andion E, *et al*: Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med* **343**: 1350-1354, 2000.
13) Tanaka S, Kobayashi I, Utsuki S, *et al*: O^6-methylguanine-DNA methyltranspherase gene expression in gliomas by means of real-time quantitative RT-PCR and clinical response to nitrosoureas. *Int J Cancer* **103**: 67-72, 2003.
14) Nagane M, Kobayashi K, Ohnishi A, *et al*: Prognostic significance of O^6-methylguanine-DNA methyltransferase protein expression in patients with recurrent glioblastoma treated with temozolomide. *Jpn J Clin Oncol* **37**: 897-906, 2007.
15) Maxwell JA, Johnson SP, Quinn JA, *et al*: Quantitative analysis of O^6-alkylguanine-DNA alkyltransferase in malignant glioma. *Mol Cancer Ther* **5**: 2531-2539, 2006.
16) Silber JR, Bobola MS, Ghatan S, *et al*: O^6-methylguanine-DNA methyltransferase activity in adult gliomas: relation to patient and tumor characteristics. *Cancer Res* **58**: 1068-1073, 1998.
17) Nagane M, Asai A, Shibui S, *et al*: Expression of O^6-methylguanine-DNA methyltransferase and chloroethylnitrosourea resistance of human brain tumors. *Jpn J Clin Oncol* **22**: 143-149, 1992.
18) Belanich M, Pastor M, Randall T, *et al*: Retrospective study of the correlation between the DNA repair protein alkyltransferase and survival of brain tumor patients treated with carmustine. *Cancer Res* **56**: 783-788, 1996.
19) Hegi ME, Diserens AC, Gorlia T, *et al*: MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med* **352**: 997-1003, 2005.
20) Yip S, Iafrate AJ and Louis DN: Molecular diagnostic testing in malignant gliomas: a practical update on predictive markers. *J Neuropathol Exp Neurol* **67**: 1-15, 2008.
21) Kanamori M, Kon H, Nobukuni T, *et al*: Microsatellite instability and the PTEN1 gene mutation in a subset of early onset gliomas carrying germline mutation or promoter methylation of the hMLH1 gene. *Oncogene* **19**: 1564-1571, 2000.
22) Leung SY, Chan TL, Chung LP, *et al*: Microsatellite instability and mutation of DNA mismatch repair genes in gliomas. *Am J Pathol* **153**: 1181-1188, 1998.
23) Friedman HS, McLendon RE, Kerby T, *et al*: DNA mismatch repair and O^6-alkylguanine-DNA alkyltransferase analysis and response to Temodal in newly diagnosed malignant glioma. *J Clin Oncol* **16**: 3851-3857, 1998.
24) Alonso M, Hamelin R, Kim M, *et al*: Microsatellite instability occurs in distinct subtypes of pediatric but not adult central nervous system tumors. *Cancer Res* **61**: 2124-2128, 2001.
25) 永根基雄，大西晶子，小林啓一・他: 再発悪性神経膠腫に対するtemozolomide療法の経験および治療感受性の検討. *Neuro-Oncology* (Tokyo) **14**: 22-29, 2004.
26) Hunter C, Smith R, Cahill DP, *et al*: A hypermutation

phenotype and somatic MSH6 mutations in recurrent human malignant gliomas after alkylator chemotherapy. *Cancer Res* **66**: 3987-3991, 2006.
27) Nicholson HS, Kretschmar CS, Krailo M, *et al*: Phase 2 study of temozolomide in children and adolescents with recurrent central nervous system tumors: a report from the Children's Oncology Group. *Cancer* **110**: 1542-1550, 2007.
28) Hirose Y, Berger MS and Pieper RO: p53 effects both the duration of G2/M arrest and the fate of temozolomide-treated human glioblastoma cells. *Cancer Res* **61**: 1957-1963, 2001.
29) Hirose Y, Kreklau EL, Erickson LC, *et al*: Delayed repletion of O^6-methylguanine-DNA methyltransferase resulting in failure to protect the human glioblastoma cell line SF767 from temozolomide-induced cytotoxicity. *J Neurosurg* **98**: 591-598, 2003.
30) Hirose Y, Katayama M, Berger MS, *et al*: Cooperative function of Chk1 and p38 pathways in activating G2 arrest following exposure to temozolomide. *J Neurosurg* **100**: 1060-1065, 2004.
31) Liu L and Gerson SL: Targeted modulation of MGMT: clinical implications. *Clin Cancer Res* **12**: 328-331, 2006.
32) Quinn JA, Pluda J, Dolan ME, *et al*: Phase II trial of carmustine plus O (6)-benzylguanine for patients with nitrosourea-resistant recurrent or progressive malignant glioma. *J Clin Oncol* **20**: 2277-2283, 2002.
33) Quinn JA, Desjardins A, Weingart J, *et al*: Phase I trial of temozolomide plus O^6-benzylguanine for patients with recurrent or progressive malignant glioma. *J Clin Oncol* **23**: 7178-7187, 2005.
34) Clemons M, Kelly J, Watson AJ, *et al*: O^6-(4-bromothenyl) guanine reverses temozolomide resistance in human breast tumour MCF-7 cells and xenografts. *Br J Cancer* **93**: 1152-1156, 2005.
35) Tolcher AW, Gerson SL, Denis L, *et al*: Marked inactivation of O^6-alkylguanine-DNA alkyltransferase activity with protracted temozolomide schedules. *Br J Cancer* **88**: 1004-1011, 2003.
36) Natsume A, Ishii D, Wakabayashi T, *et al*: IFN-beta down-regulates the expression of DNA repair gene MGMT and sensitizes resistant glioma cells to temozolomide. *Cancer Res* **65**: 7573-7579, 2005.
37) Natsume A, Wakabayashi T, Ishii D, *et al*: A combination of IFN-beta and temozolomide in human glioma xenograft models: implication of p53-mediated MGMT down-regulation. *Cancer Chemother Pharmacol* **61**: 653-659, 2008.
38) Brandes AA, Basso U, Reni M, *et al*: First-line chemotherapy with cisplatin plus fractionated temozolomide in recurrent glioblastoma multiforme: a phase II study of the Gruppo Italiano Cooperativo di Neuro-Oncologia. *J Clin Oncol* **22**: 1598-1604, 2004.
39) Silvani A, Eoli M, Salmaggi A, *et al*: Phase II trial of cisplatin plus temozolomide, in recurrent and progressive malignant glioma patients. *J Neurooncol* **66**: 203-208, 2004.
40) Yung WK, Prados MD, Yaya-Tur R, *et al*: Multicenter phase II trial of temozolomide in patients with anaplastic astrocytoma or anaplastic oligoastrocytoma at first relapse. Temodal Brain Tumor Group. *J Clin Oncol* **17**: 2762-2771, 1999.
41) Cairncross JG, Ueki K, Zlatescu MC, *et al*: Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. *J Natl Cancer Inst* **90**: 1473-1479, 1998.
42) Jenkins RB, Blair H, Ballman KV, *et al*: A t (1;19) (q10; p10) mediates the combined deletions of 1p and 19q and predicts a better prognosis of patients with oligodendroglioma. *Cancer Res* **66**: 9852-9861, 2006.
43) Kouwenhoven MC, Kros JM, French PJ, *et al*: 1p/19q loss within oligodendroglioma is predictive for response to first line temozolomide but not to salvage treatment. *Eur J Cancer* **42**: 2499-2503, 2006.
44) Cairncross G, Berkey B, Shaw E, *et al*: Phase III trial of chemotherapy plus radiotherapy compared with radiotherapy alone for pure and mixed anaplastic oligodendroglioma: Intergroup Radiation Therapy Oncology Group Trial 9402. *J Clin Oncol* **24**: 2707-2714, 2006.
45) van den Bent MJ, Carpentier AF, Brandes AA, *et al*: Adjuvant procarbazine, lomustine, and vincristine improves progression-free survival but not overall survival in newly diagnosed anaplastic oligodendrogliomas and oligoastrocytomas: a randomized European Organisation for Research and Treatment of Cancer phase III trial. *J Clin Oncol* **24**: 2715-2722, 2006.
46) Furnari FB, Fenton T, Bachoo RM, *et al*: Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes Dev* **21**: 2683-2710, 2007.
47) Omuro AM, Faivre S and Raymond E: Lessons learned in the development of targeted therapy for malignant gliomas. *Mol Cancer Ther* **6**: 1909-1919, 2007.
48) Prados MD, Lamborn KR, Chang S, *et al*: Phase 1 study of erlotinib HCl alone and combined with temozolomide in patients with stable or recurrent malignant glioma. *Neuro Oncol* **8**: 67-78, 2006.
49) Marie Y, Carpentier AF, Omuro AM, *et al*: EGFR tyrosine kinase domain mutations in human gliomas. *Neurology* **64**: 1444-1445, 2005.
50) Barber TD, Vogelstein B, Kinzler KW, *et al*: Somatic mutations of EGFR in colorectal cancers and glioblastomas. *N Engl J Med* **351**: 2883, 2004.
51) Mellinghoff IK, Wang MY, Vivanco I, *et al*: Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *N Engl J Med* **353**: 2012-2024, 2005.
52) Wang MY, Lu KV, Zhu S, *et al*: Mammalian target of rapamycin inhibition promotes response to epidermal growth factor receptor kinase inhibitors in PTEN-deficient and PTEN-intact glioblastoma cells. *Cancer Res* **66**: 7864-7869, 2006.
53) Hay N: The Akt-mTOR tango and its relevance to cancer. *Cancer Cell* **8**: 179-183, 2005.
54) O'Reilly KE, Rojo F, She QB, *et al*: mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res* **66**: 1500-1508, 2006.
55) Lachat Y, Diserens AC, Nozaki M, *et al*: INK4a/Arf is required for suppression of EGFR/DeltaEGFR (2-7)-dependent ERK activation in mouse astrocytes and glioma. *Oncogene* **23**: 6854-6863, 2004.
56) Reya T, Morrison SJ, Clarke MF, *et al*: Stem cells, cancer, and cancer stem cells. *Nature* **414**: 105-111, 2001.
57) Kondo T: Brain cancer stem-like cells. *Eur J Cancer* **42**: 1237-1242, 2006.
58) Singh SK, Hawkins C, Clarke ID, *et al*: Identification of human brain tumour initiating cells. *Nature* **432**: 396-401, 2004.
59) Vescovi AL, Galli R and Reynolds BA: Brain tumour stem cells. *Nat Rev Cancer* **6**: 425-436, 2006.
60) Zheng X, Shen G, Yang X, *et al*: Most C6 cells are cancer stem cells: evidence from clonal and population analyses. *Cancer Res* **67**: 3691-3697, 2007.
61) 瀬戸口啓夫, 近藤 亨, 田賀哲也: 癌幹細胞の同定とその意義. 蛋白質 核酸 酵素 **50**: 1995-2000, 2005.
62) Michor F, Hughes TP, Iwasa Y, *et al*: Dynamics of chronic myeloid leukaemia. *Nature* **435**: 1267-1270, 2005.
63) Kang MK and Kang SK: Tumorigenesis of chemothera-

peutic drug-resistant cancer stem-like cells in brain glioma. *Stem Cells Dev* **16**: 837-847, 2007.
64) Liu G, Yuan X, Zeng Z, *et al*: Analysis of gene expression and chemoresistance of CD133+cancer stem cells in glioblastoma. *Mol Cancer* **5**: 67, 2006.
65) Salmaggi A, Boiardi A, Gelati M, *et al*: Glioblastoma-derived tumorospheres identify a population of tumor stem-like cells with angiogenic potential and enhanced multidrug resistance phenotype. *Glia* **54**: 850-860, 2006.
66) Bao S, Wu Q, McLendon RE, *et al*: Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444**: 756-760, 2006.
67) Dean M, Fojo T and Bates S: Tumour stem cells and drug resistance. *Nat Rev Cancer* **5**: 275-284, 2005.
68) Lee J, Son MJ, Woolard K, *et al*: Epigenetic-mediated dysfunction of the bone morphogenetic protein pathway inhibits differentiation of glioblastoma-initiating cells. *Cancer Cell* **13**: 69-80, 2008.
69) Clement V, Sanchez P, de Tribolet N, *et al*: HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity. *Curr Biol* **17**: 165-172, 2007.
70) Nakano I, Saigusa K and Kornblum HI: BMPing off glioma stem cells. *Cancer Cell* **13**: 3-4, 2008.

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Synergistic cytotoxicity through the activation of multiple apoptosis pathways in human glioma cells induced by combined treatment with ionizing radiation and tumor necrosis factor–related apoptosis-inducing ligand

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Object. Malignant gliomas remain incurable despite modern multimodality treatments. Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL), also known as Apo2L, a member of the TNF family, preferentially induces apoptosis in human tumor cells through its cognate death receptors DR4 or DR5, suggesting that it may serve as a potential therapeutic agent for intractable malignant gliomas. Here, the authors show that genotoxic ionizing radiation synergistically enhances TRAIL-induced cell death in human glioma cells expressing DR5.

Methods. Combination treatment with soluble human TRAIL plus radiation induced robust cell death, while each of them singly led to only limited cytotoxicity. The combination resulted in cleavage and activation of the apoptotic initiator caspase-8 and the effector caspase-3 as well as cleavage of Bid and another initiator caspase-9, a downstream component of the apoptosome. Accordingly, it augmented the release of cytochrome c from the mitochondria into the cytosol, as well as apoptosis-inducing factor. Synergistic cell death was suppressed by TRAIL-neutralizing DR5-Fc, caspase inhibitors, expression of dominant-negative Fas-associated protein with death domain and CrmA, which selectively blocks caspase-8, and overexpression of Bcl-X_L. Finally, combination treatment had no influence on the viability of normal human astrocytes.

Conclusions. These results suggest that combination treatment with TRAIL and ionizing radiation kills human glioma cells through the activation of DR5-mediated death receptor pathways. This therapy involves direct activation of effector caspases as well as mitochondria-mediated pathways and provides a novel strategy in which TRAIL could be synergistically combined with DNA-damaging radiation.

KEY WORDS • glioblastoma multiforme • DR5 • radiotherapy • apoptosis tumor necrosis factor–related apoptosis-inducing ligand

MALIGNANT glioma, the most common primary brain tumor, remains incurable despite intensive multimodality treatments including surgery, external-beam radiotherapy, and chemotherapy. The 5-year survival rate in patients with GBM, the most malignant form of glioma, has never exceeded 10%,[6] necessitating the development of novel treatment strategies. Besides the difficulty involved in complete surgical removal due to consistent infiltration of glioma cells into adjacent functional brain parenchyma, resistance to radiotherapy and chemotherapy is also an important determinant for successful treatment of malignant gliomas.

Apoptosis is a genetically controlled form of cell death that appears to be involved in tumor cell killing by most chemotherapeutic agents and by irradiation through disparate modes of action and cellular targets.[15] Death ligands interact with their cognate receptors containing DDs, and thereby directly triggering suicide signal transduction pathways that are mainly independent of mitochondrial dysfunction, one of the major targets of external apoptotic signals.[1] Also called Apo2L, TRAIL is a member of the death ligand TNF family, which includes FasL (CD95L) and TNFα, and is capable of inducing rapid apoptosis in tumor cells of diverse origins but not in most normal cells in vitro.[39,42,63] Unlike FasL and TNF, which induce acute toxicity after systemic administration (especially in the liver because of the high expression of cognate receptors in hepa-

Abbreviations used in this paper: AIF = apoptosis-inducing factor; DD = death domain; DISC = death-inducing signaling complex; DN = dominant negative; FADD = Fas-associated protein with DD; FasL = Fas ligand; GBM = glioblastoma multiforme; IR = ionizing radiation; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP = poly(adenosine diphosphate-ribose) polymerase; PVDF = polyvinylidene difluoride; RIPA = radioimmunoprecipitation buffer; TNF = tumor necrosis factor; TRAIL = TNF-related apoptosis-inducing ligand; TUNEL = terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

tocytes[16,35]), the systemic administration of soluble human TRAIL has been shown to have little toxicity in mice or nonhuman primates,[3,60] suggesting its potential value for cancer therapy. Furthermore, untagged trimeric TRAIL containing stoichiometric Zn has been shown to have no cytotoxicity in human hepatocytes or keratinocytes.[24,40]

Note that TRAIL can interact with two death receptors, DR4 (TRAIL-R1)[37] and DR5 (KILLER/TRAIL-R2/TRICK2),[36,50] and that the death signal can be blocked by the expression of the antagonistic decoy receptors DcR1 (TRID/TRAIL-R3)[36,50] and DcR2 (TRUNDD/TRAIL-R4)[8,29] and osteoprotegerin,[10] which can compete with DR4 or DR5 for binding to TRAIL. Both DR4 and DR5 transcripts are expressed to some extent in some glioma cells but at low levels in the brain.[11,36,42,50] On activation of DR4 and/or DR5, the TRAIL-induced death signal is transduced through the activation of caspases, a family of cysteine proteases that specifically cleave at aspartic acid residues and play a central role in the execution of apoptosis,[56] in a manner similar to the extrinsic FasL/Fas (Apo1/CD95)–induced apoptotic signaling pathway.[7] Oligomerization of DR4 or DR5 causes the recruitment of adaptor molecules, preferentially FADD, through their DD interaction, which in turn activates the initiator caspase-8 or -10.[1] Consequently, effector caspase-3 or -7 is directly activated and irreversible cell death processes subsequently progress. Note that TRAIL can also activate intrinsic mitochondrial apoptosis pathways to various extents where cytochrome c release from the mitochondria leads to the formation of the "apoptosome" with Apaf-1, deoxyadenosine 5′-triphosphate, and procaspase-9, resulting in the activation of an initiator caspase-9 and the subsequent progression of the caspase cascade.[14,66] Disrupting the integrity of the mitochondrial membrane potential can also trigger a release of Smac/DIABLO, AIF, or HtrA2/Omi into the cytosol, modulating the intensity of apoptosis signaling and DNA fragmentation.[7,27] The mitochondrial apoptosis pathways are mainly regulated by molecules of the Bcl-2 family, including Bid, which promotes apoptosis by interacting with antiapoptotic Bcl-2 family members on cleavage by activated caspase-8.[66]

Ionizing radiation therapy has been consistently shown to be a favorable prognostic factor for malignant gliomas[6,18] and is applied postoperatively in most cases. Ionizing irradiation induces double-strand breaks in DNA, thereby affecting mitochondrial integrity, which leads to activation of the downstream caspase cascade and apoptosis. Damage to DNA has been shown to upregulate DR5 expression in some human cancer cells,[49,64] and we have previously demonstrated that treatment with genotoxic chemotherapeutic agents upregulated DR5 transcription and thereby induced synergistic cytotoxicity in combination with TRAIL in the majority of human glioma cell lines tested.[33]

These observations prompted us to test whether DNA-damaging IR could enhance TRAIL-induced glioma cell death. Here we show that IR induced an increase in DR5, but not DR4, protein expression, which per se did not lead to apoptosis of human glioma cell lines. However, when combined with soluble human TRAIL, IR caused a dramatic and synergistic cell death and suppressed clonogenic survival. Mechanistically, the cytotoxicity induced by the combination treatment required FADD and caspase-8, and the mitochondrial apoptosis pathway was also involved through Bid cleavage.

Materials and Methods

Reagents Used

Soluble human recombinant FLAG-TRAIL was prepared as previously described[33] and was stored at −80°C. Soluble chimeric fusion proteins and DR5-Fc and its Fc control were prepared as described.[36] The anti-FLAG monoclonal antibody M2 was obtained from Sigma. A broad-spectrum peptide caspase inhibitor, z-Asp-CH_2-DCB, and a caspase-9 inhibitor, z-LEHD-FMK, were purchased from Bachem and BD PharMingen, respectively, and were prepared in dimethyl sulfoxide.

Plasmid Constructs and Virus Preparation

The CrmA expression virus vector was constructed in pBabePuro using the EcoRI fragment of p1843DNA (a gift from Dr. D. Pickup).[12] The pBp.CrmA virus was produced by cotransfecting 293 cells with the pBp.CrmA and the plasmid pHCMV-G encoding vesicular stomatitis virus G glycoprotein,[65] and supernatant was harvested 3 days after the transfection. Virus-containing supernatant was concentrated by centrifugation to obtain high-titer retrovirus.[65] The pBp virus was also produced as an empty virus control.

Tumor Cells

The human GBM cell lines used were described previously.[59] The A1207 cell line was a gift from Dr. S. Aaronson. The T98G cells were infected with either pBp.CrmA or the pBp control virus and then grown in the presence of 500 ng/ml puromycin (Calbiochem), and a bulk population of cells expressing high levels of CrmA was used for the experiments. The T98G cells were transfected with either pcDNA3.DN-FADD plasmid or its control vector, pcDNA3 plasmid (gifts from Dr. G. Pan and Dr. C. Kitanaka, respectively), by using the calcium phosphate precipitation method and were selected in the presence of 1200 μg/ml G418 (Gibco/BRL). Clones expressing high levels of DN-FADD were used for experiments. The T98G cells expressing high levels of Bcl-X_L were also established by transfection with pSFFVneo-bcl-X_L plasmid or its control vector, pSFFV-neo plasmid (gifts from Dr. S. J. Korsmeyer), as described earlier in this paper. All cells were cultured as previously described.[32]

Radiation Therapy

Exponentially growing cells were trypsinized and plated in either 96-well or 12-well plates overnight. The cells were then irradiated in an MBR-1505R2 x-ray source (Hitachi Medical Corp.) at various doses as indicated, followed immediately by treatment with TRAIL in a fresh medium until subsequent assays.

Western Blotting

Whole-cell lysates were prepared in RIPA buffer and subjected to Western blot analysis, as previously described.[32] Proteins on the PVDF membranes were probed with antibodies against DR5 (polyclonal, R & D Systems), DR4 (polyclonal, BD PharMingen), DcR2 (polyclonal, Imgenex), FADD (monoclonal, BD Transduction), Bax (Ab-1, NeoMarkers), Bak (Ab-1, Oncogene), Bid (polyclonal, BD PharMingen), Bcl-2 (Ab-1, NeoMarkers), Bcl-X_L (polyclonal, BD Transduction), caspase-8 (polyclonal, BD PharMingen), caspase-9 (monoclonal, Trevigen), caspase-3 and HtrA2 (polyclonal, gifts from Dr. R. Takahashi), PARP (C2-10, Enzyme System Products), cytochrome c (monoclonal, BD PharMingen), AIF (monoclonal, Santa Cruz Biotechnology), Smac (polyclonal, Cell Signaling Technology), β-actin (monoclonal, Sigma), or CrmA (polyclonal, gift from Dr. D. Pickup), and were detected by chemiluminescence.

Growth Inhibition Assays

Cytotoxicity was evaluated using the MTT survival assays as described.[30] Briefly, cells were plated at 10^4 cells/well in 96-well microtiter plates overnight. The cells were then treated with 200 μl fresh medium containing drugs, cultured for 24 hours, treated with 250 μg/ml MTT for an additional 4 hours, and analyzed using a microplate reader (Molecular Devices). The effects of treatment were

expressed as a percentage of growth inhibition, using untreated cells as the uninhibited control.

Apoptosis Assay

Assays of cell death were performed using in situ cell death detection kits (Roche) or TUNEL, as described.[32] The apoptotic index was calculated as the ratio of apoptotic cell number to total tumor cell number after examination of more than 1000 cells.

Clonogenic Assay

Colony-forming efficiency was tested, as described.[31] Briefly, approximately 500 cells were plated in triplicate into six-well plates and cultured overnight. The cells were then irradiated and treated with TRAIL. After further incubation for 10 to 14 days, they were fixed and colonies of 50 or more cells were scored. The survival fraction was calculated as the ratio of the colony-forming efficiency of treated to untreated cells.

Preparation of Cytosolic and Mitochondrial Proteins

The cells were harvested by centrifugation at 1800 G for 5 minutes at 4°C. After being washed once with ice-cold phosphate-buffered saline, the cell pellets were resuspended in 5 vols of ice-cold buffer A (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium ethylenediaminetetraacetic acid, 1 mM sodium ethyleneglycoltetraacetic acid, and 1 mM dithiothreitol) containing 250 mM sucrose supplemented with protease inhibitors. After sitting on ice for 15 minutes, the cells were disrupted by douncing 15 times in a 5-ml dounce homogenizer with the B pestle. The homogenates were centrifuged twice at 750 G for 10 minutes at 4°C. The supernatant was centrifuged at 10,000 G for 25 minutes at 4°C, and the resulting pellets were resuspended in lysis (RIPA) buffer, as described earlier (mitochondrial fraction). Supernatant recovered from the 10,000-G spin was further centrifuged at 100,000 G for 1 hour at 4°C, and the resulting supernatant (the cytosolic extract) was recovered and stored at −80°C.

Statistical Analysis

The data were analyzed for significance using the Student t-test. Synergism between TRAIL and IR was assessed using a software program (CalcuSyn, Biosoft) based on median dose-effect analysis.[5] The combination index was calculated for a two-drug combination involving a fixed concentration ratio. With these methods, a combination index less than 1.0 indicated a synergistic interaction.

Results

Ionizing Radiation Synergistically Enhances TRAIL-Mediated Cytotoxicity in Vitro in Human Glioma Cells

We sought to determine whether genotoxic IR could enhance TRAIL-induced cytotoxicity in glioma cells. One-time irradiation at various doses had little effect on cell viability in human glioma cell lines more than 48 hours after treatment, although it did reduce clonogenic growth in a dose-dependent manner as expected (data not shown). A low concentration (0.1 μg/ml) of human soluble FLAG-TRAIL also had only marginal cytotoxicity. However, when this low concentration of TRAIL was combined with a single dose of 10 Gy of radiation, substantial and synergistic death of T98G cells was evident within 48 hours. This reaction was accompanied by changes in structure typical of apoptotic cell death (Fig. 1A), which was confirmed by DNA fragmentation detected using TUNEL assays (Fig. 1B). A median dose-effect analysis of the cytotoxicity caused by the combination treatment over a range of IR (2.5–20 Gy) and TRAIL (0.05–0.4 μg/ml) doses determined using MTT assays yielded combination index values considerably less than 1.0 (combination index 0.109), especially at higher doses of both treatments, which corresponds to a strong synergism (Fig. 1C). Furthermore, clonogenic assays demonstrated a high inhibition rate of clonogenic survival on treatment with the combination of TRAIL and even a subtoxic dose of IR (2 Gy) in T98G cells, compared with each of the single treatments (Fig. 1D). Similarly enhanced cytotoxicity by combined treatment with IR and TRAIL was observed in the other human p53-mutated glioma cell lines U251MG (Fig. 1B, D, and E), SF188, and LN428. Interestingly, this effect was not apparent in U87MG, U178MG, and A1207 glioma cell lines, which are all p53 wild type.

Ionizing Radiation Induces Expression of DR5/TRAIL-R2 in Human Glioma Cells

To explore the underlying mechanisms by which IR augments TRAIL cytotoxicity in human glioma cells, we next determined whether radiation could increase the expression of TRAIL receptors. Results of Western blot analysis showed that the basal level expression of the 55-kD DR5 protein increased significantly in a time-dependent manner after treatment with IR at a dose of 10 Gy in the glioma cell lines T98G, U251MG, and LN428, which had responded to the combination treatment (Fig. 2A). In T98G cells, the expression level of the DR5 protein at 48 hours after IR was 3.9-fold higher than that without irradiation, whereas that of another cell death–inducing TRAIL receptor, DR4, did not change on irradiation (1.1-fold). Irradiation resulted in only a marginal increase in the decoy TRAIL receptor DcR2 (1.6-fold). Similarly, DR5 protein expression increased 1.9- and 2.1-fold on irradiation in U251MG and LN428 cells, respectively, which showed comparatively smaller enhancement of TRAIL-induced cytotoxicity by irradiation than the T98G cells. In contrast, the application of 10 Gy of radiation resulted in no appreciable increases in any of the TRAIL receptors in the U87MG cells, which had not responded to the combination treatment.

Irradiation at 10 Gy led to the increased expression of other apoptosis-related molecules such as Bak (1.7-fold) and procaspase-3 (2.3-fold) in T98G cells, and Bax (1.8-fold) in LN428 cells, indicating that irradiation might augment TRAIL sensitivity by modifying the expression of molecules involved in multiple intracellular apoptosis pathways (Fig. 2B).

Combined IR and TRAIL Induces Activation of Multiple Caspases

Having determined that DR5 protein expression increased in human glioma cell lines on exposure to IR, we next determined whether the radiation-enhanced TRAIL cytotoxicity could be mediated through caspase activation, a hallmark process of apoptotic execution. Whereas one-time exposure to either radiation or TRAIL led to little if any cleavage of caspase-8, an initiator caspase at the apex of the caspase activation cascade, in T98G or U251MG cells, respectively, combination treatment resulted in marked cleavage of caspase-8 (Fig. 3A). Similarly, 32 kDa of caspase-3, a major effector caspase, was significantly cleaved following combination treatment, as was PARP, an intracellular substrate of activated caspase-3. Furthermore, combination treatment resulted in cleavage of Bid, which

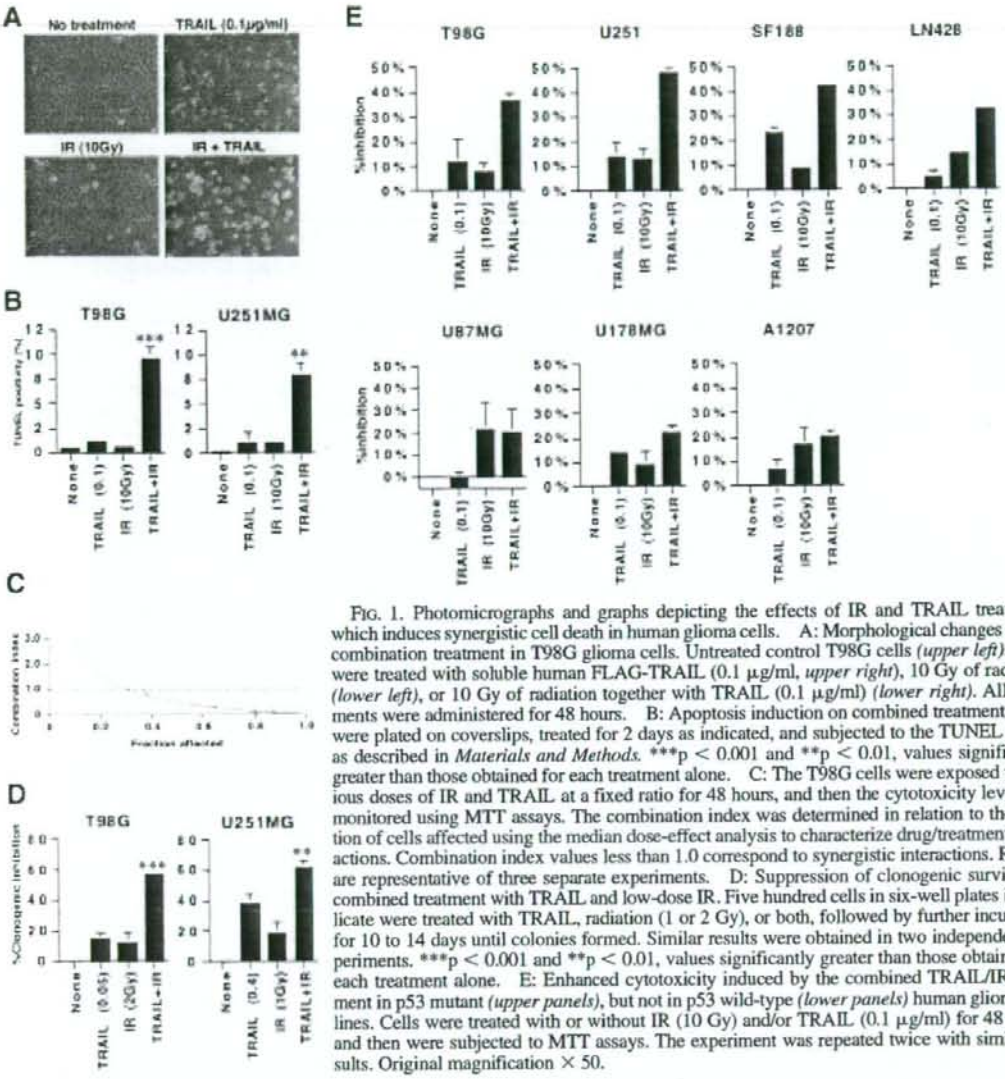

FIG. 1. Photomicrographs and graphs depicting the effects of IR and TRAIL treatment, which induces synergistic cell death in human glioma cells. A: Morphological changes on the combination treatment in T98G glioma cells. Untreated control T98G cells *(upper left)*. Cells were treated with soluble human FLAG-TRAIL (0.1 μg/ml, *upper right*), 10 Gy of radiation *(lower left)*, or 10 Gy of radiation together with TRAIL (0.1 μg/ml) *(lower right)*. All treatments were administered for 48 hours. B: Apoptosis induction on combined treatment. Cells were plated on coverslips, treated for 2 days as indicated, and subjected to the TUNEL assay, as described in *Materials and Methods*. ***$p < 0.001$ and **$p < 0.01$, values significantly greater than those obtained for each treatment alone. C: The T98G cells were exposed to various doses of IR and TRAIL at a fixed ratio for 48 hours, and then the cytotoxicity level was monitored using MTT assays. The combination index was determined in relation to the fraction of cells affected using the median dose-effect analysis to characterize drug/treatment interactions. Combination index values less than 1.0 correspond to synergistic interactions. Results are representative of three separate experiments. D: Suppression of clonogenic survival by combined treatment with TRAIL and low-dose IR. Five hundred cells in six-well plates in triplicate were treated with TRAIL, radiation (1 or 2 Gy), or both, followed by further incubation for 10 to 14 days until colonies formed. Similar results were obtained in two independent experiments. ***$p < 0.001$ and **$p < 0.01$, values significantly greater than those obtained for each treatment alone. E: Enhanced cytotoxicity induced by the combined TRAIL/IR treatment in p53 mutant *(upper panels)*, but not in p53 wild-type *(lower panels)* human glioma cell lines. Cells were treated with or without IR (10 Gy) and/or TRAIL (0.1 μg/ml) for 48 hours, and then were subjected to MTT assays. The experiment was repeated twice with similar results. Original magnification × 50.

would be expected to activate the mitochondrial apoptosis pathway. These cleavages of caspase-3 and -8, Bid, and PARP were inhibited in the presence of the TRAIL-neutralizing chimeric soluble receptor DR5-Fc, suggesting that binding of TRAIL to DR5 could mediate the enhanced caspase activation induced by the combination treatment.

As expected, on combined IR/TRAIL treatment leading to cleavage of the BH3-containing Bcl-2 family member Bid, cleavage of caspase-9, a key initiator caspase involved in the mitochondrial apoptosis pathway (Fig. 3B), was also apparent. Caspase activation and PARP cleavage were abrogated in the presence of the broad caspase inhibitor z-Asp-CH_2-DCB, whereas a caspase-9 selective inhibitor, z-LEHD-FMK, also partially suppressed cleavage of caspase-3 and PARP. Consistently, the pretreatment of T98G cells with these caspase inhibitors significantly suppressed the cytotoxicity induced by the combination treatment, where z-Asp-CH_2-DCB appeared the more potent inhibitor than z-LEHD-FMK (Fig. 3C). These results suggested a role for mitochondria in cell-death induction by the combination treatment.

Both FADD and Caspase-8 are Required for the Synergistic Cytotoxicity Induced by Combined IR/TRAIL Treatment

We next determined whether the death signal induced by IR/TRAIL stems from the activation of the apical caspase

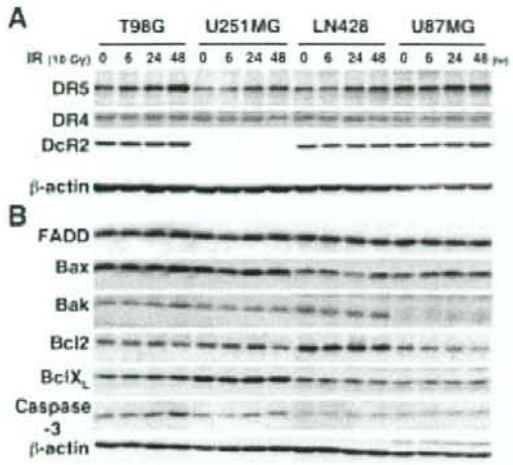

FIG. 2. Gel blots showing glioma cell line reactions to IR. A: Induction of DR5 protein expression by IR in a time-dependent manner in human glioma cell lines. Cells were irradiated with x-rays at 10 Gy for the indicated hours (0, 6, 24, and 48) and harvested for preparation of total lysates. Forty micrograms of total lysate was size-fractionated in a 12.5% sodium dodecyl sulfate–polyacrylamide gel, transferred to a PVDF membrane, and induced to react sequentially with polyclonal antibodies against human DR5, DR4, and DcR2. The β-actin blot demonstrates loading of lysate in each lane. B: Protein expression of other apoptosis-related molecules on application of IR. Western blot analyses were performed using the specific antibodies indicated.

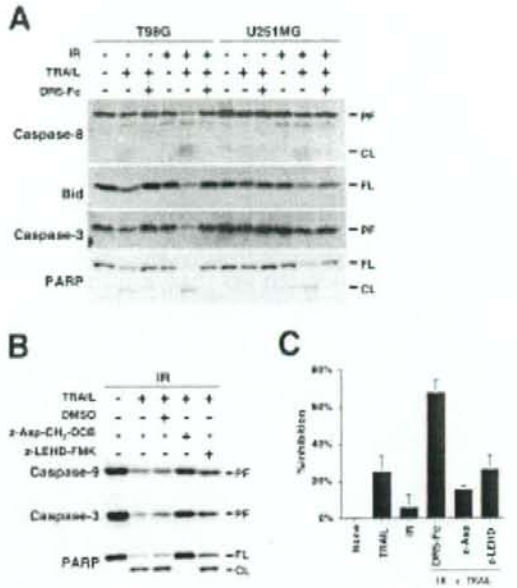

FIG. 3. Gel blots and graph demonstrating the effects of combined treatment with IR and TRAIL, which induces multiple caspase activation in human glioma cells. A: Activation of caspase-8 and -3 by administering the combined treatment in T98G and U251MG cells. Substrates of caspase-8 and -3, Bid and PARP, respectively, are also significantly cleaved on the administration of combined IR/TRAIL, which are completely abrogated in the presence of the TRAIL-neutralizing chimeric protein DR5Fc. B: Activation of caspase-9 with the administration of the combined treatment in T98G cells. Effector caspase-3 activation is totally suppressed by a broad caspase inhibitor, z-Asp-CH_2-DCB, and treatment with a caspase-9 inhibitor, z-LEHD-FMK, results in partial suppression of caspase-3 activation. C: Synergistic cytotoxicity induced by the combined treatment is suppressed by caspase inhibitors in T98G cells. Cells were treated with or without IR (10 Gy) and/or TRAIL (0.1 μg/ml) in the presence or absence of DR5Fc (A), z-Asp-CH_2-DCB (100 μM), or z-LEHD-FMK (20 μM, B and C) for 48 hours and then were either harvested to prepare total lysates for Western blot analysis (A and B) or subjected to MTT assay (C). CL = cleaved form; FL = full length; PF = proform.

pathway and the formation of the DISC. We generated subclones of T98G cells stably expressing a DN-FADD (Fig. 4A). Although combined treatment with IR and TRAIL induced synergistic cytotoxicity in the control cells transfected with the empty vector (T98.pcDNA3), such an effect was not apparent in cells expressing high amounts of DN-FADD and was also significantly reduced in those with its relatively low expression (Fig. 4B). Similarly, we generated T98G cells expressing the virus protein CrmA, a selective caspase-8 inhibitor (Fig. 4C). Expression of CrmA also resulted in significant suppression of IR/TRAIL-induced cytotoxicity (Fig. 4D). These results suggested that FADD and caspase-8 are required for the synergistic cell death induced by the combination treatment and activation of the apical caspase cascade plays an essential role in death signal induction. Similar results were observed in U251MG cells expressing DN-FADD or CrmA (data not shown).

Involvement of Mitochondrial Damage in the IR/TRAIL Treatment–Induced Cytotoxicity

We next determined the effects of the combination treatment on mitochondria. Treatment with either IR or TRAIL led to a slight elevation in the mitochondrial proteins cytochrome c, AIF, and Smac in the cytosol. The combination treatment further increased the cytosolic release of these proteins (Fig. 5A), suggesting that mitochondrial damage is enhanced on combined cytotoxic treatment, which is consistent with the caspase-9 activation described earlier. This effect appeared to have some specificity because the cytosolic fraction of HtrA2/Omi, another mitochondrial apoptosis-related molecule, was somewhat reduced on both TRAIL treatment alone and combined IR/TRAIL (Fig. 5A).

We also generated subclones of T98G cells stably expressing Bcl-X_L (Fig. 5B). Overexpression of Bcl-X_L resulted in complete suppression of the cytotoxicity induced by IR, TRAIL, or both (Fig. 5C), further supporting the argument for the involvement of mitochondrial damage in cell death.

Combined IR/TRAIL Treatment Does not Induce Cell Death in Normal Human Astrocytes

We next examined the effects of the treatments on normal human astrocytes. Treatment with IR (10 and 20 Gy) or TRAIL (0.1 μg/ml) alone and their combination did not cause cytotoxicity in these cells. In contrast, T98G cells, the positive control for the combined treatment, did show the

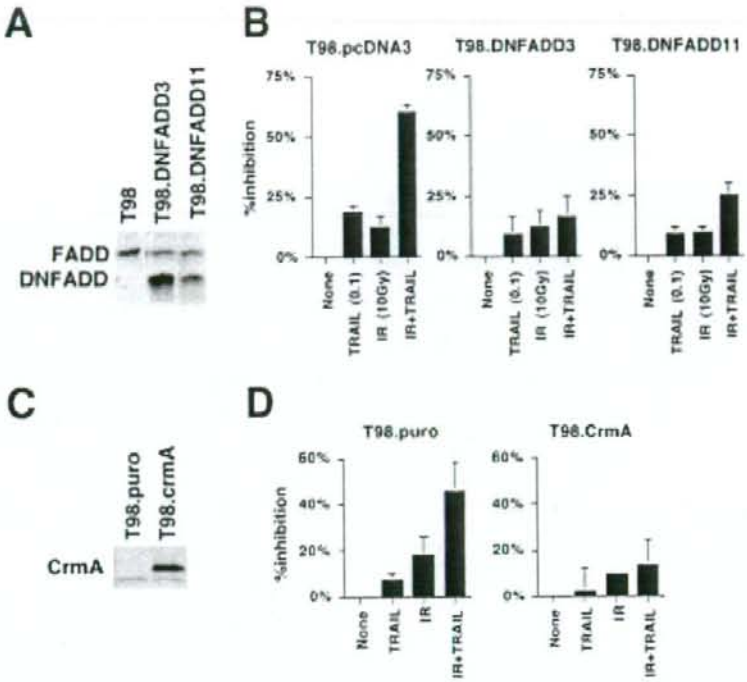

FIG. 4. Both FADD and caspase-8 are required in synergistic cytotoxicity induced by the combined IR/TRAIL treatment. A: Gel blot showing T98G and its subclones expressing varying amounts of DN-FADD (DNFADD) along with wild-type endogenous FADD. B. Bar graphs demonstrating inhibition of the enhanced cytotoxicity induced by the combination treatment in T98G cells expressing DN-FADD in a dose-dependent manner. Cells were treated with or without IR (10 Gy) and/or TRAIL (0.1 μg/ml) for 48 hours and then were subjected to MTT assay. C: Gel blot showing T98G cells were infected with either a retrovirus encoding CrmA or an empty virus, and cell populations stably expressing CrmA were selected during puromycin treatment. D: Graphs revealing inhibition of enhanced cytotoxicity induced by the combination treatment in T98G cells expressing CrmA, a selective virus protein-inhibiting caspase-8. Cells were treated with or without IR (20 Gy) and/or TRAIL (0.1 μg/ml) for 48 hours and then were subjected to MTT assay.

expected enhancement of cytotoxicity (Fig. 6), indicating that normal human astrocytes are insensitive to the combined treatment with these agents.

Discussion

Malignant gliomas are the most common malignant neoplasms of the central nervous system and show extensive infiltration into surrounding brain parenchyma, rendering surgical extirpation incomplete and thus necessitating adjuvant therapies to treat residual tumor cells. External-beam radiotherapy has been accepted as a standard modality for postoperative treatment and has been shown to increase the survival period in patients with malignant gliomas.[61,62] However, the survival benefit that radiotherapy can bestow in such patients is limited to 4 to 6 months at the most,[22,61,62] suggesting the existence of many radioresistant gliomas. Several approaches to enhance the radiation responsiveness of malignant gliomas have been tried, including the utilization of chemical radiosensitizers such as nitroimidazole compounds to hypoxic tumor cells[41,57] and halogenated pyrimidine analogs to nonhypoxic tumor cells.[43] Nonetheless, results from clinical trials have failed to demonstrate significant benefit from the addition of these drugs to radiotherapy for GBM,[38] although some authors have shown improvement in survival in patients harboring anaplastic astrocytoma with the combined treatment of pyrimidine analogs and radiation.[26,58] The use of most chemotherapeutic agents for cancer has shown no radiosensitization in patients with malignant glioma,[53] suggesting that new strategies to enhance the efficacy of radiotherapy must be pursued.

The induction of apoptosis through death receptor–mediated intracellular signaling is an intriguing anticancer strategy, especially because it involves signaling pathways that directly activate the death-executing caspase cascade rather than the mitochondrial damage through which most current anticancer therapies, including IR, operate.[20] Here, we show that the combination of IR and soluble TRAIL treatment resulted in significant enhancement of cytotoxicity in human glioma cells through rapid induction of apoptosis. During the first 48 hours after application, IR upregulated DR5 protein expression in glioma cells that showed enhanced sensitivity to the treatment, and the upregulation of DR5 expres-

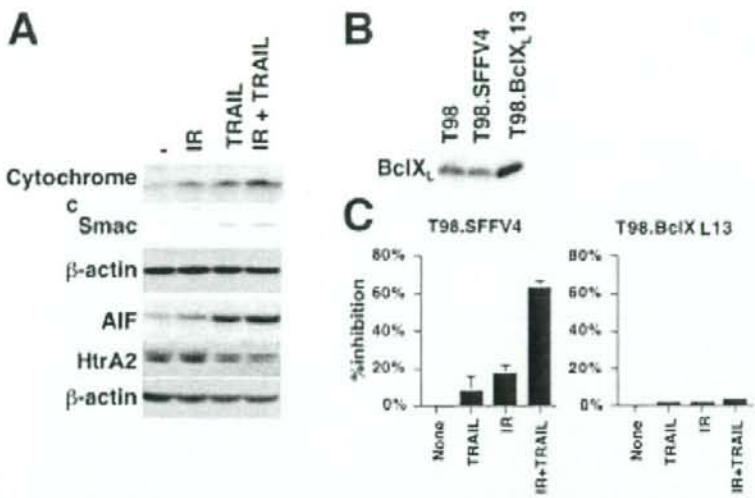

FIG. 5. Gel blots and graphs showing involvement of mitochondrial apoptosis pathways on treatment with combined IR and TRAIL. A: Cytosolic release of mitochondrial proteins on combination treatment with IR and TRAIL. The T98G cells were treated with or without radiation (10 Gy) and/or TRAIL (0.1 μg/ml) for 48 hours, and cytosolic fractions were prepared as described in *Materials and Methods*. Cytosolic proteins were subjected to Western blot analysis using the antibodies indicated. The β-actin blots demonstrate loading of lysate in each lane. B and C: The Bcl-X_L inhibits synergistic apoptosis induction by the combined treatment of IR and TRAIL. Western blot analysis shows overexpression of Bcl-X_L in T98G.BclX_L13 cells as compared with T98G parental and empty-vector control T98G.SFFV4 cells (B). Enhanced cytotoxicity induced by the combination treatment is abrogated in T98G.BclX_L13 cells. Cells were treated with or without IR (20 Gy) and/or TRAIL (0.1 μg/ml) for 48 hours and then were subjected to MTT assay.

sion and TRAIL receptor interaction were essential in this effect given that TRAIL-neutralizing DR5-Fc abolished the cytotoxicity induced by the combination treatment. In this sense, IR could represent a sensitizing therapy for death-inducing soluble TRAIL treatment as well as soluble TRAIL's action as a radiosensitizer.

Among the DD-positive receptors for TRAIL, it was only DR5, and not DR4, whose protein expression was specifically upregulated by IR, whereas expression levels of the decoy TRAIL receptor DcR2 remained unaffected in glioma cells. This DR5 preponderance was consistent with our previous observations that genotoxic chemotherapeutic agents synergized with TRAIL in apoptotic induction through upregulation of DR5 but not other TRAIL receptors.[33] Moreover, the majority of human glioma cell lines tested underwent apoptosis after treatment with agonistic anti–human DR5 monoclonal human antibodies, whereas no cell lines responded to an agonistic anti–human DR4 monoclonal human antibody (unpublished data). These results suggest that DR5 could represent the crucial TRAIL receptor in human glioma. In contrast to DR5, the role of other key molecules in the TRAIL-induced apoptosis signaling pathways—including FADD, Bax, and Bak[52]—in the sensitization to TRAIL appeared to be marginal, because their modulation by radiation was inconsistent in glioma cell lines.

The IR-induced DR5 upregulation and subsequent enhancement of TRAIL-mediated cytotoxicity in glioma cells appears to be p53 independent given that most human glioma cell lines whose DR5 expression was upregulated by IR (and so were significantly sensitive to the IR/TRAIL combination treatment) actually harbor *TP53* mutations. Additionally, p53 wild-type U87MG cells were insensitive to the combined treatment with no change in the DR5 expression level on IR therapy. Similarly, U178MG and A1207 cells, which are also wild type for *TP53*, showed only minor or no enhanced cytotoxicity, respectively, on combination treatment. Furthermore, suppression of wild-type p53 function by the exogenous expression of p53DD, a DN form of p53, failed to sensitize U87MG cells to the treatment (data not shown). This result is in contrast to our observation that other genotoxic chemotherapeutic agents induced synergistic cytotoxicity with TRAIL in all p53 wild-type glioma cells tested.[33] Taken together, wild-type p53 function may be irrelevant to the efficacy of the combined IR/TRAIL treatment in human glioma cells, providing benefit for its clinical application because nearly half of malignant gliomas harbor *TP53* mutations. The involvement of p53 function in the irradiation-induced DR5 upregulation and augmented TRAIL sensitivity might be cell type–dependent, given that p53 dependence was demonstrated in cancer cells from the breast,[4] prostate,[47] and blood (leukemia).[13] Indeed, both p53-dependent and -independent regulation of DR5 expression has been demonstrated in cell type– and stimulation-specific manners,[49] suggesting the possibility of multiple regulatory pathways leading to transactivation of DR5 expression.

Soluble TRAIL activates the extrinsic receptor-mediated pathway where DD-containing adaptor molecules and initiator caspases are recruited to activate DR4 or DR5 for DISC formation.[46] In the IR/TRAIL-sensitive human glioma cells, inhibition of FADD by the overexpression of its DN form resulted in a remarkable abrogation of the cyto-

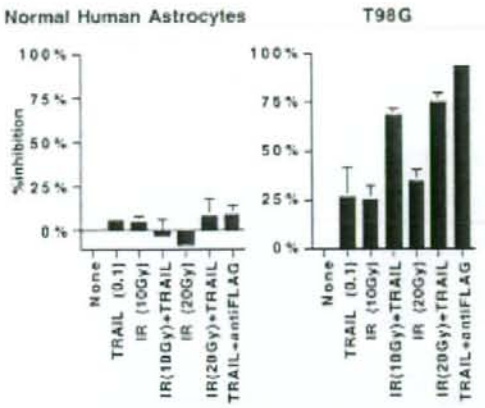

FIG. 6. Bar graphs demonstrating that combined treatment with IR and TRAIL does not affect the viability of normal human astrocytes. Cells were treated with or without IR (10 or 20 Gy) and/or TRAIL (0.1 μg/ml) for 48 hours and then were subjected to MTT assays. Treatment with TRAIL and anti-FLAG antibody was included as a positive control for the assay. The experiment was repeated twice with similar results.

toxic effect with the combination treatment. Expression of CrmA, which selectively inhibits caspase-8 activation,[9] also suppressed synergistic cell death on combination therapy, indicating a requirement for FADD and caspase-8 in this synergistic death-inducing signaling as the key adaptor molecule and initiator caspase, respectively. This result is consistent with the TRAIL-mediated extrinsic pathway observed in other cell types as well as glioma.[44,52] These observations further support a role for irradiation as a sensitizer of TRAIL,[48] because this apical caspase activation is generally absent in radiation-induced cytotoxicity. Marini et al.[28] reported that caspase-10 was required in leukemic cells, which lack caspase-8, in the synergistic response to IR and TRAIL, suggesting that caspase-10 may also transduce augmented death signals at its apical phase. In glioma, our observations of caspase-8 expression in all cell lines tested and complete suppression of the synergy by the caspase-8 selective inhibitor CrmA suggest that caspase-8 may play a central role in the cytotoxicity of the combined therapy in glioma cells.

Suppression of the combined IR/TRAIL treatment–induced cytotoxicity by overexpression of Bcl-X_L indicates a substantial involvement of mitochondria in the apoptosis pathways in glioma cells, because Bcl-X_L exerts its antiapoptotic action mainly by preventing permeabilization of the outer mitochondrial membrane.[14,23] Activation of the intrinsic mitochondrial apoptotic pathways was further demonstrated by the release of the mitochondrial molecules cytochrome c and Smac into the cytosol, which resulted in activation of the apoptosomal caspase-9. Note that the enhanced release of another mitochondrial apoptosis-promoting factor, AIF (which directly activates nuclear DNA damage in a caspase-independent fashion), may also contribute to the cytotoxicity of the combined treatment and may account for the only partial inhibition of apoptosis by the caspase-9 inhibitor z-LEHD compared with the strong suppression by Bcl-X_L expression. In contrast to AIF, the cytosolic fraction of HtrA2/Omi, which binds to the X-linked inhibitor of apoptosis and promotes caspase activation,[54] was reduced on IR/TRAIL treatment, suggesting specificity in the response. The underlying basis of the differential release of these mitochondrial proteins remains to be elucidated. Extensive cleavage of Bid by the caspase-8 activation on treatment with IR and TRAIL suggests that mitochondrial impairment was induced, at least in part, through the cross-talk from the extrinsic receptor-mediated pathway as well as the initial direct damage caused by IR. The extent of mitochondrial involvement in the IR/TRAIL-induced apoptosis may vary by cell type, given that the overexpression of another antiapoptotic molecule, Bcl-2, failed to protect leukemia cells from cytotoxicity induced by the combination treatment.[28]

The low sensitivity of human glioma cells to the sole treatment with the FLAG-tagged soluble TRAIL might be related to this particular formulation of human soluble TRAIL, because FLAG-TRAIL required antibody-mediated cross-linking for maximum activity, whereas other versions of human soluble TRAIL, such as His-tagged or the untagged trimeric version of human TRAIL, transduce apoptosis without additional cross-linkers.[34,40,42,45] In this sense, the use of trimeric soluble TRAIL would generate more potent cytotoxic effects at lower concentrations in vivo where cross-linkers are unavailable. There is also a formulation-dependent difference in the TRAIL-induced toxicity profile. A preparation of His-tagged TRAIL, consisting of multimeric aggregates, exerted strong hepatocyte toxicity,[19] and the membrane-bound form of TRAIL induced liver injury.[17] Furthermore, the His-tagged version or a soluble form fused to trimerizing leucine zipper (LZ-TRAIL) have been shown to induce apoptosis on normal keratinocytes.[25,40] In contrast, the untagged soluble version, comprising the amino acid residues 114 to 281, showed no apoptotic effect on the same type of cells.[40] This difference might be due to the over-multimerization of the TRAIL death receptors by the tagged preparations of TRAIL, because such preparations may induce aggregation and precipitation at higher concentrations. The receptor over-multimerization could then lead to a signal surpassing the high threshold for apoptosis activation in normal cells.[2] The untagged zinc-bound TRAIL may not trigger such an apoptotic signal in normal cells because of its highly stable and soluble nature as a trimer.[2,34] Therefore, this version of TRAIL would be expected to demonstrate the optimal toxicity profile in clinical settings. Nevertheless, our results demonstrate that the tagged soluble form of TRAIL synergizes with IR to preferentially kill GBM cells with mutant forms of p53.

In addition to the suppression of clonogenic survival by combining TRAIL with low-dose radiation, the enhanced cell death induced by the combination treatment reached maximum levels at a dose of 10 Gy. This result suggests that combining TRAIL with stereotactic radiosurgery might also lead to effective killing of tumor cells, as could combining TRAIL with conventional fractionated external-beam radiotherapy. Indeed, authors of several reports have shown that the use of radiosurgery as a radiation dose boost after the completion of conventional fractionated radiotherapy could improve survival in patients with malignant glioma.[21,46,51] The radiosurgical approach might also be applica-

ble for recurrent gliomas that have previously been treated with conventional radiotherapy.[55] Taken together, our results showed that the combination of TRAIL and IR causes rapid glioma cell death and suppresses clonogenic survival without affecting normal astrocytes. This result may provide a potential basis for the application of this therapy as a novel strategy against intractable gliomas, even on recurrence, by utilizing high-energy radiotherapy and thus warrants further investigation in animal models and in clinical settings with the untagged trimeric version of human TRAIL.[24,40]

Conclusions

Combined treatment with the apoptosis-inducing death ligand TRAIL and IR, the well-established standard therapeutic modality for malignant glioma, kills human glioma cells through the activation of DR5-mediated death receptor pathways, which involves direct activation of not only effector caspases but also mitochondria-mediated pathways. This result suggests that a therapeutic strategy for malignant gliomas in which TRAIL could be synergistically combined with DNA-damaging radiotherapy might be possible.

Acknowledgments

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References

1. Almasan A, Ashkenazi A: Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy. **Cytokine Growth Factor Rev 14:**337–348, 2003
2. Ashkenazi A: Targeting death and decoy receptors of the tumor-necrosis factor superfamily. **Nat Rev Cancer 2:**420–430, 2002
3. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, et al: Safety and antitumor activity of recombinant soluble Apo2 ligand. **J Clin Invest 104:**155–162, 1999
4. Chinnaiyan AM, Prasad U, Shankar S, Hamstra DA, Shanaiah M, Chenevert TL, et al: Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. **Proc Natl Acad Sci U S A 97:**1754–1759, 2000
5. Chou TC, Talalay P: Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. **Adv Enzyme Regul 22:**27–55, 1984
6. The Committee of Brain Tumor Registry of Japan: Report of Brain Tumor Registry of Japan (1969–1996). **Neurol Med Chir (Tokyo) 43 Suppl,** 2003
7. Danial NN, Korsmeyer SJ: Cell death: critical control points. **Cell 116:**205–219, 2004
8. Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA, Goodwin RG: The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. **Immunity 7:**813–820, 1997
9. Ekert PG, Silke J, Vaux DL: Caspase inhibitors. **Cell Death Differ 6:**1081–1086, 1999
10. Emery JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C, et al: Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. **J Biol Chem 273:**14363–14367, 1998
11. Frank S, Köhler U, Schackert G, Schackert HK: Expression of TRAIL and its receptors in human brain tumors. **Biochem Biophys Res Commun 257:**454–459, 1999
12. Furnari FB, Lin H, Huang HS, Cavenee WK: Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. **Proc Natl Acad Sci U S A 94:**12479–12484, 1997
13. Gong B, Almasan A: Apo2 ligand/TNF-related apoptosis-inducing ligand and death receptor 5 mediate the apoptotic signaling induced by ionizing radiation in leukemic cells. **Cancer Res 60:** 5754–5760, 2000
14. Green DR, Reed JC: Mitochondria and apoptosis. **Science 281:** 1309–1312, 1998
15. Guchelaar HJ, Vermes A, Vermes I, Haanen C: Apoptosis: molecular mechanisms and implications for cancer chemotherapy. **Pharm World Sci 19:**119–125, 1997
16. Havell EA, Fiers W, North RJ: The antitumor function of tumor necrosis factor (TNF), I. Therapeutic action of TNF against an established murine sarcoma is indirect, immunologically dependent, and limited by severe toxicity. **J Exp Med 167:**1067–1085, 1988
17. Ichikawa K, Liu W, Zhao L, Wang Z, Liu D, Ohtsuka T, et al: Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. **Nat Med 7:**954–960, 2001
18. Jelsma R, Bucy PC: The treatment of glioblastoma multiforme of the brain. **J Neurosurg 27:**388–400, 1967
19. Jo M, Kim TH, Seol DW, Esplen JE, Dorko K, Billiar TR, et al: Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. **Nat Med 6:**564–567, 2000
20. Kaufmann SH, Earnshaw WC: Induction of apoptosis by cancer chemotherapy. **Exp Cell Res 256:**42–49, 2000
21. Kondziolka D, Flickinger JC, Bissonette DJ, Bozik M, Lunsford LD: Survival benefit of stereotactic radiosurgery for patients with malignant glial neoplasms. **Neurosurgery 41:**776–785, 1997
22. Kristiansen K, Hagen S, Kollevold T, Torvik A, Holme I, Nesbakken R, et al: Combined modality therapy of operated astrocytomas grade III and IV. Confirmation of the value of postoperative irradiation and lack of potentiation of bleomycin on survival time: a prospective multicenter trial of the Scandinavian Glioblastoma Study Group. **Cancer 47:**649–652, 1981
23. Kuwana T, Newmeyer DD: Bcl-2-family proteins and the role of mitochondria in apoptosis. **Curr Opin Cell Biol 15:**691–699, 2003
24. Lawrence D, Shahrokh Z, Marsters S, Achilles K, Shih D, Mounho B, et al: Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. **Nat Med 7:**383–385, 2001
25. Leverkus M, Neumann M, Mengling T, Rauch CT, Brocker EB, Krammer PH, et al: Regulation of tumor necrosis factor-related apoptosis-inducing ligand sensitivity in primary and transformed human keratinocytes. **Cancer Res 60:**553–559, 2000
26. Levin VA, Silver P, Hannigan J, Wara WM, Gutin PH, Davis RL, et al: Superiority of post-radiotherapy adjuvant chemotherapy with CCNU, procarbazine, and vincristine (PCV) over BCNU for anaplastic gliomas: NCOG 6G61 final report. **Int J Radiat Oncol Biol Phys 18:**321–324, 1990
27. Lorenzo HK, Susin SA: Mitochondrial effectors in caspase-independent cell death. **FEBS Lett 557:**14–20, 2004
28. Marini P, Jendrossek V, Durand E, Gruber C, Budach W, Belka C: Molecular requirements for the combined effects of TRAIL and ionising radiation. **Radiother Oncol 68:**189–198, 2003
29. Marsters SA, Sheridan JP, Pitti RM, Huang A, Skubatch M, Baldwin D, et al: A novel receptor for Apo2L/TRAIL contains a truncated death domain. **Curr Biol 7:**1003–1006, 1997
30. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. **J Immunol Methods 65:**55–63, 1983
31. Nagane M, Asai A, Shibui S, Nomura K, Kuchino Y: Application of antisense ribonucleic acid complementary to O6-methylguanine-deoxyribonucleic acid methyltransferase messenger ribonucleic acid for therapy of malignant gliomas. **Neurosurgery 41:** 434–441 1997
32. Nagane M, Coufal F, Lin H, Bögler O, Cavenee WK, Huang HJS: A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. **Cancer Res 56:**5079–5086, 1996

33. Nagane M, Pan G, Weddle JJ, Dixit VM, Cavenee WK, Huang HJS: Increased death receptor 5 expression by chemotherapeutic agents in human gliomas causes synergistic cytotoxicity with tumor necrosis factor-related apoptosis-inducing ligand *in vitro* and *in vivo*. **Cancer Res 60:**847–853, 2000
34. Nagata S: Apoptosis by death factor. **Cell 88:**355–365, 1997
35. Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, et al: Lethal effect of the anti-Fas antibody in mice. **Nature 364:**806–809, 1993
36. Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM: An antagonist decoy receptor and a death domain-containing receptor for TRAIL. **Science 277:**815–818, 1997
37. Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, et al: The receptor for the cytotoxic ligand TRAIL. **Science 276:** 111–113, 1997
38. Phillips TL, Levin VA, Ahn DK, Gutin PH, Davis RL, Wilson CB, et al: Evaluation of bromodeoxyuridine in glioblastoma multiforme: a Northern California Cancer Center Phase II study. **Int J Radiat Oncol Biol Phys 21:**709–714, 1991
39. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, 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
40. Qin J, Chaturvedi V, Bonish B, Nickoloff BJ: Avoiding premature apoptosis of normal epidermal cells. **Nat Med 7:**385–386, 2001
41. Rabin HR, Urtasun RC, Partington J, Koziol D, Sharon M, Walker K: High-dose metronidazole: pharmacokinetics and bioavailability using an iv preparation and application of its use as a radiosensitizer. **Cancer Treat Rep 64:**1087–1095, 1980
42. Rieger J, Naumann U, Glaser T, Ashkenazi A, Weller M: APO2 ligand: a novel lethal weapon against malignant glioma? **FEBS Lett 427:**124–128, 1998
43. Rodriguez R, Ritter MA, Fowler JF, Kinsella TJ: Kinetics of cell labeling and thymidine replacement after continuous infusion of halogenated pyrimidines in vivo. **Int J Radiat Oncol Biol Phys 29:**105–113, 1994
44. Rohn TA, Wagenknecht B, Roth W, Naumann U, Gulbins E, Krammer PH, et al: CCNU-dependent potentiation of TRAIL/Apo2L-induced apoptosis in human glioma cells is p53-independent but may involve enhanced cytochrome c release. **Oncogene 20:**4128–4137, 2001
45. Roth W, Isenmann S, Naumann U, Kugler S, Bahr M, Dichgans J, et al: Locoregional Apo2L/TRAIL eradicates intracranial human malignant glioma xenografts in athymic mice in the absence of neurotoxicity. **Biochem Biophys Res Commun 265:**479–483, 1999
46. Sarkaria JN, Mehta MP, Loeffler JS, Buatti JM, Chappell RJ, Levin AB, et al: Radiosurgery in the initial management of malignant gliomas: survival comparison with the RTOG recursive partitioning analysis. Radiation Therapy Oncology Group. **Int J Radiat Oncol Biol Phys 32:**931–941, 1995
47. Shankar S, Singh TR, Srivastava RK: Ionizing radiation enhances the therapeutic potential of TRAIL in prostate cancer in vitro and in vivo: intracellular mechanisms. **Prostate 61:**35–49, 2004
48. Shankar S, Srivastava RK: Enhancement of therapeutic potential of TRAIL by cancer chemotherapy and irradiation: mechanisms and clinical implications. **Drug Resist Updat 7:**139–156, 2004
49. Sheikh MS, Burns TF, Huang Y, Wu GS, Amundson S, Brooks KS, et al: p53-dependent and -independent regulation of the death receptor KILLER/DR5 gene expression in response to genotoxic stress and tumor necrosis factor alpha. **Cancer Res 58:** 1593–1598, 1998
50. Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, et al: Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. **Science 277:**818–821, 1997
51. Shrieve DC, Alexander E III, Black PM, Wen PY, Fine HA, Kooy HM, et al: Treatment of patients with primary glioblastoma multiforme with standard postoperative radiotherapy and radiosurgical boost: prognostic factors and long-term outcome. **J Neurosurg 90:**72–77, 1999
52. Song JH, Song DK, Pyrzynska B, Petruk KC, Van Meir EG, Hao C: TRAIL triggers apoptosis in human malignant glioma cells through extrinsic and intrinsic pathways. **Brain Pathol 13:** 539–553, 2003
53. Steel G: Combined radiotherapy-chemotherapy: principles, in Steel GG, Adams GE, Horwich E (eds): **The Biological Basis of Radiotherapy, ed 2.** Amsterdam: Elsevier, 1989, p 267
54. Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R: A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. **Mol Cell 8:** 613–621, 2001
55. Tatter SB: Recurrent malignant glioma in adults. **Curr Treat Options Oncol 3:**509–524, 2002
56. Thornberry NA, Lazebnik Y: Caspases: enemies within. **Science 281:**1312–1316, 1998
57. Urtasun R, Band P, Chapman JD, Feldstein ML, Mielke B, Fryer C: Radiation and high-dose metronidazole in supratentorial glioblastomas. **N Engl J Med 294:**1364–1367, 1976
58. Urtasun RC, Cosmatos D, DelRowe J, Kinsella TJ, Lester S, Wasserman T, et al: Iododeoxyuridine (IUdR) combined with radiation in the treatment of malignant glioma: a comparison of short versus long intravenous dose schedules (RTOG 86–12). **Int J Radiat Oncol Biol Phys 27:**207–214, 1993
59. Van Meir EG, Kikuchi T, Tada M, Li H, Diserens AC, Wojcik BE, et al: Analysis of the p53 gene and its expression in human glioblastoma cells. **Cancer Res 54:**649–652, 1994
60. Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, et al: Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. **Nat Med 5:**157–163, 1999
61. Walker MD, Alexander E Jr, Hunt WE, MacCarty CS, Mahaley MS Jr, Mealey J Jr, et al: Evaluation of BCNU and/or radiotherapy in the treatment of anaplastic gliomas. A cooperative clinical trial. **J Neurosurg 49:**333–343, 1978
62. Walker MD, Green SB, Byar DP, Alexander E Jr, Batzdorf U, Brooks WH, et al: Randomized comparisons of radiotherapy and nitrosoureas for the treatment of malignant glioma after surgery. **N Engl J Med 303:**1323–1329, 1980
63. Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, et al: Identification and characterization of a new member of the TNF family that induces apoptosis. **Immunity 3:**673–682, 1995
64. Wu GS, Burns TF, McDonald ER III, Jiang W, Meng R, Krantz ID, et al: KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. **Nat Genet 17:**141–143, 1997
65. Yee JK, Miyanohara A, LaPorte P, Bouic K, Burns JC, Friedmann T: A general method for the generation of high-titer, pantropic retroviral vectors: highly efficient infection of primary hepatocytes. **Proc Natl Acad Sci U S A 91:**9564–9568, 1994
66. Younes A, Kadin ME: Emerging applications of the tumor necrosis factor family of ligands and receptors in cancer therapy. **J Clin Oncol 21:**3526–3534, 2003

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Original Articles

Prognostic Significance of O^6-Methylguanine-DNA Methyltransferase Protein Expression in Patients with Recurrent Glioblastoma Treated with Temozolomide

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Background: Temozolomide (TMZ) is active against newly diagnosed glioblastoma (GBM), and O^6-methylguanine-DNA methyltransferase (MGMT) is implicated in resistance to TMZ and nitrosoureas. We evaluated the efficacy and safety of the standard 5-day TMZ regimen in patients with recurrent GBM after initial therapy including nitrosourea-based chemotherapy, in conjunction with an analysis of the prognostic value of MGMT protein expression regarding response to TMZ and survival.

Methods: From September 2003 to January 2007, 30 patients having recurrent GBM received 150–200 mg/m^2/day of TMZ for five consecutive days every 28 days. Tumor tissue from 19 patients was analysed for MGMT protein expression using western blotting, and 17 of them were assessable for a response.

Results: The overall response rate was 23.5% (one complete response and three partial responses). Six patients had stable disease (35.3%). Median progression-free survival (PFS) time was 2.2 months, and median overall survival (OS) time was 9.9 months from the initiation of TMZ therapy. Patients with low MGMT protein expression had a significantly improved PFS ($P = 0.016$) and OS ($P = 0.019$) compared to those with high expression. Both low MGMT expression ($P = 0.040$) and re-resection at relapse ($P = 0.014$) persisted as significant independent favorable prognostic factors for OS. The most common grade 3 and 4 hematological toxicity was lymphopenia (22.2%).

Conclusions: The standard 5-day TMZ regimen resulted in moderate antitumor activity with an acceptable safety profile in patients with nitrosourea-pretreated recurrent GBM, and protein expression of MGMT is an important prognostic factor for patients treated with TMZ even after recurrence.

Key words: glioblastoma – temozolomide – O^6-methylguanine-DNA methyltransferase – western blot – recurrence

INTRODUCTION

Patients with malignant gliomas, the most common primary neoplasms arising in the central nervous system, have yet a poor prognosis, despite multimodal intensive treatments including maximum surgical resection, irradiation and chemotherapy. The median survival time (MST) of patients with glioblastoma multiforme (GBM), the most malignant form of glioma, remains 12–15 months from initial diagnosis (1). This disappointing outcome results, at least in part,

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Abbreviations: MST, medial survival time; GBM, glioblastoma multiforme; TMZ, temozolomide; EORTC, European Organisation for Research and Treatment of Cancer; NCIC, National Cancer Institute of Canada; RT, radiotherapy; MGMT, O^6-methylguanine-DNA methyltransferase; PCR, polymerase-chain reaction; MSP, methylation-specific PCR; IHC, immunohistochemistry; KPS, Karnofsky performance status; MRI, magnetic resonance imaging; PFS, progression-free survival; OS, overall survival; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

from low responsiveness to chemotherapy. The therapeutic benefit of chemotherapy using nitrosoureas, alkylating agents such as carmustine, lomustine and nimustine, has been controversial, even in combination with procarbazine, lomustine and vincristine (PCV), until the Medical Research Council reported a large-scale meta-analysis of 12 selected randomized clinical trials demonstrating a significant improvement in risk of progression and survival (2). However, the survival benefit remained unsatisfactory, with only a 15% reduction in the hazard ratio.

Temozolomide (TMZ) is an alkylating agent that is rapidly absorbed after oral administration, and penetrates well into the cerebrospinal fluid at a concentration up to 40% of that measured in plasma (3–6). TMZ has been shown to have clinical antitumor activity against malignant gliomas and a relatively good safety profile (7,8). A recent phase III clinical trial conducted by the European Organisation for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) demonstrated that concomitant radiotherapy (RT) plus TMZ followed by six cycles of TMZ significantly prolonged survival at the hazard ratio of 65% compared with RT alone in patients with newly diagnosed GBM (9). The RT plus concomitant and adjuvant TMZ regimen has thus been considered as the new standard care for this group of patients. However, no standard of care exists for recurrent GBM yet.

The mechanism of action by TMZ is thought to be methylation at the O^6 position of guanine in DNA, with additional methylation at the N^7 position (10,11). These alkylating lesions are effectively repaired by a DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT). MGMT catalyzes the stoichiometric, covalent transfer of the alkyl group to an internal cysteine residue, and is finally inactivated (12). A direct relationship between MGMT activity or expression and resistance to alkylating nitrosoureas, which also exert their antitumor effects by alkylating the O^6 position of guanine, has been well documented in cell lines and xenografts derived from a variety of human tumors, including gliomas (13,14). Likewise, a significant correlation was recently found between methylation status of the *MGMT* gene promoter determined by the methylation-specific polymerase-chain reaction (PCR) (MSP) assay and survival of patients with newly diagnosed GBM treated with RT plus concomitant and adjuvant TMZ (15). The methylation of the promoter region of *MGMT* gene has been shown to be a major mechanism to turn off gene transcription, thus is speculated to reduce the intracellular level of MGMT expression (16). MGMT protein expression determined by immunohistochemistry (IHC) was also found to correlate with survival or response to TMZ treatment in newly diagnosed GBM patients (17,18). However, other clinical studies failed to show any relationship between *MGMT* promoter methylation status and survival or response to TMZ, even for dose-intensified regimens, in patients with recurrent GBM (19,20). These contradictory results could be due to the timing of TMZ administration, i.e. at initial diagnosis versus at recurrence, or might be dependent on assay methods for detection of MGMT status (21,22).

Herein, we report the efficacy and safety of the standard 5-day on/28-day cycle regimen of TMZ in patients with recurrent GBM after failure of multimodal intensive initial therapy including nitrosourea-based chemotherapy. In addition, MGMT protein expression determined by western blotting was analysed for a correlation with response to TMZ and survival.

MATERIALS AND METHODS

Patient Eligibility

Patients were eligible for this study if they had been diagnosed with recurrent GBM. All patients were ≥3 years of age with a Karnofsky performance status (KPS) ≥60. They had to show objective evidence of tumor recurrence or progression and to have adequate hematologic, renal and hepatic function as follows: absolute neutrophil count ≥1500/μl, platelet count ≥100 000/μl, hemoglobin ≥8 g/dl, serum creatinine and bilirubin levels less than 1.5 times the upper limit of laboratory normal and AST or ALT ≤3 times the upper limit of laboratory normal. Patients were required to have a life expectancy greater than 3 months, to have prior chemotherapy or radiation 4 weeks or more before, and to have provided written informed consent. The treatment protocol was approved by the Institutional Ethics Committees.

Treatment

TMZ was provided by Schering-Plough Corp. It was administered for a maximum of 30 cycles or until unacceptable toxicity or tumor progression occurred. Patients received TMZ (150 mg/m^2/day) for 5 days every 4 weeks in the first cycle (750 mg/m^2/cycle). In the absence of grade 3 or 4 hematologic toxicity, dosing for the following cycle could be increased to 200 mg/m^2/day for 5 days (1000 mg/m^2/cycle). Repeat cycles were administered on schedule only if, before the first day of the next cycle, grade 2 or greater neutropenia or thrombocytopenia was absent. In the case of grade 3 or greater hematologic toxicity or reversible grade 3 non-hematologic toxicity (except for nausea/vomiting), the dose of TMZ was reduced by 25%, with the lowest dose being 100 mg/m^2/day. In cases of non-hematologic grade 4 toxicity, chemotherapy was interrupted. Prophylactic anti-emetics were given routinely. Neurologic stability was provided with the lowest corticosteroid dose when required.

Patient Evaluation

Gadolinium-enhanced magnetic resonance imaging (MRI) or contrast-enhanced computed tomography was performed at either Kyorin University Hospital or the original investigator's institution. Response evaluation conformed to the

RECIST system (23) for tumor size, and also to Macdonald's criteria (24). Patients were closely monitored for toxicity in all cycles and graded according to National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. Hematologic and biochemical evaluations were repeated at least once per cycle.

Clinical Specimens

Malignant glial tumors were surgically removed at either Kyorin University Hospital or Tokyo Metropolitan Fuchu Hospital and stored at −80°C prior to use. Patient material was obtained with informed consent on approval from the Institutional Ethics Committees.

Western Blot Analysis

Whole lysates from tumor specimens were prepared in RIPA buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1× complete protease inhibitor (Roche), 5 mg/ml pepstatin, 2 mM sodium orthovanadate, 0.5 M sodium fluoride and 0.2 M sodium pyrophosphate] and subjected to western blot analyses as previously described (25). Proteins on PVDF membranes were probed with a monoclonal antibody against MGMT (MT3.1, NeoMarker), and β-actin (monoclonal, Sigma), and were detected by chemiluminescence and quantified (LAS 1000, Fuji). The loading of lysates on membranes was evaluated by β-actin blotting.

Assessment of MGMT Protein Expression Level (MGMT Score)

MGMT protein expression was detected and quantified by western blotting as described earlier. MGMT expression was normalized relative to the β-actin level of the tumor. Tumor 'MGMT score (%)' was calculated by standardizing the MGMT level of each tumor relative to that of the T98G glioma cell line, which expresses a high level of MGMT protein and is resistant to TMZ and nitrosourea (26).

Statistical Analysis

PFS was evaluated from the first day of treatment to relapse, progression or death, or to the last date of follow-up, and overall survival (OS) was calculated as MST from the first day of treatment of the recurrent tumor to death for any reason or to the last date of follow-up. PFS and OS were calculated according to the Kaplan–Meier method, and differences in progression and survival according to prognostic factors were evaluated with the log-rank test. Parameters possibly correlated with disease progression and survival were age, gender, KPS at relapse, frontal lobe localization, presence or absence of re-resection at relapse, prior use of nitrosourea, response to treatment and expression of MGMT protein. A multivariate analysis with the Cox model, used to assess truly independent prognostic factors, was performed only for variables for which $P < 0.1$ was obtained in the univariate analysis. For continuous variables, the cut-off level chosen for KPS and MGMT score was the median value, and that for age was the mean value. All the probability values were two-sided, and all statistical analyses were done at a significance level of $P = 0.05$, using the statistical package SPSS 15.0J (SPSS, Inc., Chicago, IL, USA). The correlation of tumor MGMT protein expression with clinical variables was evaluated by the chi-square test and the Fisher's exact test. The length of follow-up was described as the median and range.

RESULTS

Patients' Characteristics

From September 2003 to January 2007, a total of 30 patients with recurrent GBM were treated with TMZ. Among them, 19 patients (11 male and 8 female, a mean age of 48.2 years) were analysed for tumor MGMT status and were included in this study; their characteristics are outlined in Table 1. One patient was lost to follow-up after initiation of treatment cycle 2 and was not assessable for response, survival and safety. Another patient was not assessable for response because of no residual tumor on MRI after re-resection. All patients had undergone surgical resection as well as RT at the time of initial diagnosis. Seventeen patients (89.5%) had received nitrosourea-based chemotherapy prior to initiation of TMZ therapy. Re-resection was performed in 10 patients (52.6%) when the tumor relapsed.

MGMT Protein Expression Analysis

The MGMT status of the tumors was analysed in 19 patients (63.3%) whose frozen tumor tissues were available using western blotting. The MGMT protein level was determined by comparing it with that of the T98G human glioma cell line having high MGMT expression and nitrosourea resistance, and was calculated as the 'MGMT score' as described in 'Materials and Methods'. The level of MGMT protein in the GBMs varied substantially (Fig. 1), ranging from almost undetectable (MGMT score 3.4) to very high (104), with a median value of 12.8 (Table 1). MGMT protein expression was not related to any clinical variables (Fisher's exact test) (Table 2). Their characteristics were similar to those of the entire patient population.

Among the 19 patients in this study, there were six patients whose primary and recurrent tumor specimens were available for MGMT protein analysis. In four cases (67%), MGMT expression was at a similar level in samples obtained from primary and recurrent tumors of the same patients. In two cases (33%), however, it increased from a low to a high level, suggesting that recurrent tumor might have resulted from selection of tumor cells expressing high levels of MGMT in a minority of cases.

Table 1. Characteristics of patients with GBM and MGMT analysis ($n = 19$)

Characteristic	Number of patients	Percentage
Sex		
Male	11	57.9
Female	8	42.1
Age, years		
Mean	48.2	
Range	7–71	
Karnofsky performance status		
Median	70	
Range	60–100	
Localization		
Frontal only	7	36.8
Other	12	63.2
Prior treatment		
Surgery at initial diagnosis	19	100
Radiotherapy	19	100
Chemotherapy	18	94.7
Re-resection at relapse (at temozolomide)	10	52.6
Prior nitrosourea chemotherapy	17	89.5
MGMT protein expression (MGMT score)		
Median	12.8	
Range	3.4–104	
High expression ($\geq$12.8)	9	
Low expression ($<$12.8)	10	

GBM, glioblastoma; MGMT, O^6-methylguanine-DNA methyltransferase; CENU, chloroethylnitrosourea.

Table 2. Characteristics of patients with glioblastoma in relation to tumor MGMT protein expression determined by western blot analysis

Variable	MGMT protein expression ($n = 19$)		
	High ($n = 9$)	Low ($n = 10$)	P value[a]
Age (year)			
$\geq$50	6	6	1.000
$<$50	3	4	
Gender			
Male	7	4	0.170
Female	2	6	
Karnofsky performance status			
$\geq$80	2	6	0.170
$<$80	7	4	
Frontal localization			
Yes	2	5	0.350
No	7	5	
Re-resection at relapse			
Yes	4	6	0.656
No	5	4	
Prior nitrosourea chemotherapy			
Yes	8	9	1.000
No	1	1	
Response			
CR + PR	1	3	0.576
SD + PD	7	6	

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.
[a]Indicates Fisher's exact test

Response

Of 19 patients whose tumor MGMT expression was determined, 17 patients were assessable for response. One had a complete response (CR) (5.9%), three had a partial response (PR) (17.6%; overall response: 23.5%), six had stable disease (SD) (35.3%) and seven had progressive disease (PD) (35.3%). TMZ treatment resulted in an overall disease control rate (CR + PR + SD) of 58.8%. All responses were confirmed mostly after a 1-month interval or at least 2 months apart. The response rates of this subpopulation were almost identical to those of the overall patient population. In the univariate analysis, none of the categoric variables was found to be significantly correlated with response (CR + PR) or overall disease control (CR + PR + SD). MGMT score tended to be lower in the responders (patients with CR/PR) than non-responders (those with SD/PD), but the difference was not significant (Mann–Whitney U-test) (Fig. 2), perhaps due to an insufficient number of patients.

Figure 1. Western blot analysis of protein expression of O^6-methylguanine-DNA methyltransferase (MGMT) in human malignant gliomas. Total lysates were prepared from surgically resected human glioblastomas and subjected to western blot analysis for MGMT. MGMT protein was detected at 25 kD in most tumors at various levels. β-Actin expression is shown as a loading control. Lysate of human glioma T98G cells was used as a positive control for MGMT expression. Numbers below the panel indicate MGMT score in each tumor. GBM, glioblastoma.

Progression-free Survival

After a median follow-up of 7.1 months (range: 2.4 – 16.7), one patient (5.6%) remained free of progression. PFS from the initiation of TMZ therapy for the 18 assessable patients in the MGMT analysis was 2.2 months (95% CI: 0.0–5.6 months).

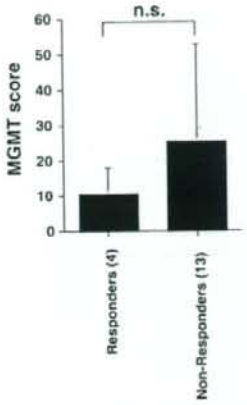

Figure 2. Relationship between the protein level in glioblastomas and clinical response.

In the univariate analysis (Table 3), only low MGMT expression was found to be significantly correlated with PFS ($P = 0.016$) (Fig. 3A). Unlike age, KPS, frontal localization and re-resection at relapse, response to treatment (CR + PR) ($P = 0.070$) was nearly significant. In the multivariate analysis (Table 4), low MGMT expression remained as the only independent prognostic factor close to the significant level ($P = 0.060$).

Overall Survival

Four of the 19 patients (21.1%) remained alive at the end of follow-up. MST from the initiation of TMZ therapy for the patients in the MGMT analysis was 9.9 months (95% CI: 5.5–14.3 months).

In the univariate analysis (Table 3), re-resection at relapse ($P = 0.002$) and low MGMT expression ($P = 0.019$) were found to be significantly correlated with OS (Fig. 3B). KPS (≥ 80 versus < 80) was near to the level of significance ($P = 0.09$), unlike age, gender, frontal localization and response to treatment. Both re-resection at relapse and KPS also remained as significant factors when analysed in the entire patient population. In the multivariate analysis (Table 4), only re-resection at relapse ($P = 0.014$) and low MGMT expression ($P = 0.040$) persisted as significant independent prognostic factors. These factors were not associated with each other (chi-square: $P = 0.463$). Because most patients had received prior nitrosourea-based chemotherapy, there was no correlation between survival and history of nitrosourea use.

There were 10 patients whose recurrent tumors were available for MGMT protein analysis. The median value for the MGMT score of these tumors was 10.2, which was slightly lower than that of all 19 tumors analysed (12.8). Using this median value, patients with low MGMT protein expression still had nearly significantly improved OS than those with high expression (log-rank, $P = 0.050$), despite a small number of patients analysed.

Table 3. Variables related to PFS and MST in patients with glioblastoma with MGMT analysis: univariate analysis

Variable	PFS (months)			MST (months)		
	Median	95% CI	*P* value[a]	Median	95% CI	*P* value[a]
Age (year)						
≥50	1.8	0.0–3.7	0.28	9.0	5.4–12.5	0.77
<50	4.4	0.0–10.0		10.4	3.3–17.5	
Gender						
Male	2.2	0.0–4.7	0.54	7.0	4.0–10.0	0.43
Female	3.4	0.0–7.9		10.3	9.5–11.2	
Karnofsky performance status						
≥80	1.7	1.5–1.9	0.41	10.3	9.2–11.4	0.09
<80	4.0	0.6–7.4		6.7	3.6–9.8	
Frontal localization						
Yes	3.4	0.0–4.7	0.18	10.3	9.5–11.2	0.65
No	2.2	0.0–7.9		7.0	4.7–9.3	
Re-resection at relapse						
Yes	1.8	1.5–2.1	0.44	10.4	10.2–10.7	0.002
No	3.4	0.0–7.1		6.7	4.4–9.0	
Prior nitrosourea chemotherapy						
Yes	1.0	0.0–5.4	0.59	9.9	5.4–14.4	0.66
No	2.2	NA		4.6	NA	
Response						
CR + PR	4.4	1.3–7.5	0.070	7.0	3.8–10.1	0.70
SD + PD	1.7	1.5–1.9		9.0	4.4–13.6	
MGMT score						
≥12.8	1.6	0.6–2.7	0.016	7.0	3.8–10.1	0.019
<12.8	4.5	2.6–6.4		10.3	8.9–11.7	

PFS, progression-free survival; MST, median survival time; CI, confidence interval; NA, not applicable.
[a]Indicates log-rank.

Toxicity

A total of 107 treatment cycles of TMZ were administered (median: six cycles per patient; range: 1–13). Treatment was generally well tolerated (Table 5). Among 18 patients who received more than one treatment cycle, the dose was escalated to 200 mg/m^2/day in all patients. Two patients received a reduction in dose to 150 mg/m^2/day and one patient, to 100 mg/m^2/day, due to nausea or infection. Regarding hematologic toxicity, grade 4 neutropenia or thrombocytopenia did not occur. Grade 4 lymphopenia was found in only one patient (5.6%). A total of five incidents of grade 3

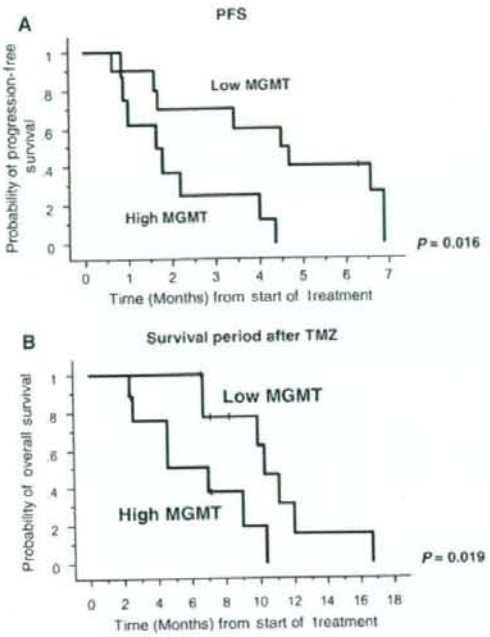

Figure 3. Kaplan–Meier estimates of (A) progression-free survival and (B) overall survival according to the MGMT. PFS, progression-free survival; TMZ, temozolomide.

hematologic toxicity were found in four patients (neutropenia: $n = 1$; thrombocytopenia: $n = 1$; lymphopenia: $n = 3$). No patients received a blood transfusion. The most common, probably treatment-related, hematologic toxicity was lymphopenia (grade 1–4, 77.8%). The incidence of non-hematologic grade 3 or 4 toxicity was low, including elevated transamidases levels (22.2%), nausea/vomiting (11.1%) and fatigue (5.6%). Two patients (11.1%) discontinued treatment because of adverse events, which consisted of grade 3 liver dysfunction ($n = 1$) and grade 2 pneumonia ($n = 1$). This pulmonary event resolved without sequelae with standard treatment. *Pneumocystis carinii* pneumonia was not confirmed in any patient despite that the prophylactic trimethoprim was not administered. There was no significant correlation between tumor MGMT protein expression and toxicity, except for grade 3 and 4 lymphopenia that occurred only in patients whose GBM expressed little MGMT protein.

DISCUSSION

Despite recent progress in the medical management of many malignant tumors, high-grade glioma, especially its most malignant form GBM, remains incurable and patients with GBM usually do not survive more than 1 year, necessitating the development of new active agents. TMZ, an orally administrable alkylating agent, has become the first significantly active therapeutic drug against newly diagnosed GBM, extending survival time by a median value of 2.7 months when concomitantly combined with conventional fractionated RT of 60 Gy, compared with RT alone (9). The survival benefits derived from the concomitant TMZ therapy were shown to correlate with methylation status of the gene of the DNA repair enzyme MGMT (15), which appeared reasonable because MGMT can sequester TMZ-induced genotoxic methyl adducts from guanines in DNA (27). These results prompted us to analyse whether TMZ could also have a similar effect on recurrent GBM, in conjunction with an analysis of the influence of tumor MGMT expression, in patients who had previously received multimodal treatment regimens including ACNU whose antitumor activity is also known to be hampered by MGMT (14,28).

In the present study, we demonstrated that the standard 5-day on/28-day cycle TMZ regimen was safe and feasible even in patients with chemotherapy-pretreated recurrent GBM. Most patients received more than two cycles of TMZ, and the major reason for the discontinuation of treatment was tumor progression, not toxicity. The TMZ therapy resulted in a MST from the initiation of therapy of 9.9 months, an OS rate at 6 months of 78.0%, a PFS rate at 6 months of 22.2%, an overall response rate of 23.5% and a disease control rate of 58.8%. These results are slightly better than those reported in patients with GBM at either first or second relapse in several phase II clinical trials, where OS at 6 months ranged from 46% to 60%, MST was 5.4–6.5 months, PFS at 6 months was 18–24%, the response rate was 8–19% and the disease control rate was 40–51% (29–32). These studies contained more patients who were chemonaïve at the initiation of TMZ therapy, suggesting that activity with the standard TMZ regimen might not be substantially affected by preceding chemotherapy with nitrosoureas.

Although molecular determinants of the sensitivity of glioma cells to TMZ may include the mismatch repair function and protein expression (18), and G2/M checkpoint proteins (33–35), MGMT has been indicated as the major factor conferring resistance to TMZ because of its direct repair of methylated guanine residues (27). As expected, patients with recurrent GBM, which expressed a low level of MGMT protein, had a significantly better OS and PFS after undergoing TMZ therapy than those with a high level of tumor MGMT expression in the univariate analyses, even though the number of patients analysed was small (Fig. 3). The low MGMT protein expression was further identified as an independent favorable prognostic factor in terms of OS, and also a nearly significant factor for PFS ($P = 0.069$) by multivariate analyses (Tables 3 and 4). These results were consistent with the findings in the large phase III EORTC/NCIC trial conducted for patients with newly diagnosed GBM (15), and further extend the notion that MGMT plays a key role in

Table 4. Predictors of PFS and overall survival in patients with glioblastoma with MGMT analysis: multivariate analysis

Variable	PFS			Overall survival		
	Hazard ratio	95% CI	*P* value[a]	Hazard ratio	95% CI	*P* value[a]
Kamofsky perfonnance status						
≥80	–			0.55	0.11–2.63	0.453
<80	–			1		
Re-resection at relapse						
Yes	–			0.11	0.02–0.64	0.014
No				1		
Response						
CR + PR	3.45	0.74–12.03	0.114	–		
SD + PD	1			–		
MGMT score						
≥12.8	3.40	0.95–12.19	0.060	4.24	1.07–16.79	0.040
<12.8	1			1		

[a]Indicates Cox model.

Table 5. Major adverse events observed in patients with GBM during all cycles of temozolomide treatment

Toxicity	Grade 3		Grade 4		Grades 1–4 total	
	Number of patients	Percentage	Number of patients	Percentage	Number of patients	Percentage
WBC count	0	0	0	0	9	50.0
Neutrophils	1	5.6	0	0	6	33.3
Platelets	1	5.6	0	0	6	33.3
Lymphocytes	3	16.7	1	5.6	14	77.8
Hemoglobin	0	0	0	0	10	55.6
Any hematologic toxicity						
Including lymphocytopenia	4	22.2	1	5.6	17	94.4
Excluding lymphocytopenia	1	5.6	0	0	14	77.8
Elevated transamidase	4	22.2	0	0	13	72.2
Nausea	2	11.1	0	0	6	33.3
Vomiting	2	11.1	0	0	3	16.7
Fatigue	0	0	1	5.6	5	27.8
Constipation	0	0	0	0	5	27.8
Epilepsy	0	0	0	0	3	16.7
Pulmonary	0	0	0	0	1	5.6
Allergic skin reaction	0	0	0	0	0	0

WBC, white blood cells.

determining the efficacy of TMZ against GBM not only in chemonaïve but also in nitrosourea-pretreated patients. Indeed, among patients whose recurrent tumors were analysed for MGMT expression, those with low MGMT expression still had improved OS than those with high expression. However, a recent phase II study by Brandes et al. (20) showed no correlation between MGMT promoter methylation status and response rate, PFS or OS in patients treated with