In vitro and In vivo Radiosensitization Induced by the DNA Methylating Agent Temozolomide

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Abstract

Purpose: Temozolomide, a DNA methylating agent, is currently undergoing clinical evaluation for cancer therapy. Because temozolomide has been shown to increase survival rates of patients with malignant gliomas when given combined with radiation, and there is conflicting preclinical data concerning the radiosensitizing effects of temozolomide, we further investigated the possible temozolomide-induced enhancement of radiosensitivity.

Experimental Design: The effects of temozolomide on the in vitro radiosensitivity of U251 (a human glioma) and MDA-MB231BR (a brain-seeking variant of a human breast tumor) cell lines was evaluated using clonogenic assay. DNA damage and repair were evaluated using phosphorylated histone H2AX (γH2AX), and mitotic catastrophe was measured using nuclear fragmentation. Growth delay was used to evaluate the effects of temozolomide on in vivo (U251) tumor radiosensitivity.

Results: Exposure of each cell line to temozolomide for 1 h before irradiation resulted in an increase in radiosensitivity with dose enhancement factors at a surviving fraction of 0.1 ranging from 1.30 to 1.32. Temozolomide had no effect on radiation-induced apoptosis or on the activation of the G2 cell cycle checkpoint. As a measure of DNA double strand breaks, γH2AX foci were determined as a function of time after the temozolomide + irradiation combination. The number of γH2AX foci per cell was significantly greater at 24 h after the combined modality compared with the individual treatments. Mitotic catastrophe, measured at 72 h, was also significantly increased in cells receiving the temozolomide + irradiation combination compared with the single treatments. In vivo studies revealed that temozolomide administration to mice bearing U251 tumor xenografts resulted in a greater than additive increase in radiation-induced tumor growth delay with a dose enhancement factor of 2.8.

Conclusions: These results indicate that temozolomide can enhance tumor cell radiosensitivity in vitro and in vivo and suggest that this effect involves an inhibition of DNA repair leading to an increase in mitotic catastrophe.

Temozolomide has known anticancer effects against a broad range of tumor histologies including gliomas (1, 2). Temozolomide is a lipophilic molecule that can be given p.o. and crosses the blood-brain barrier. At physiologic pH, temozolomide is converted to the active metabolite methyltriazenoimidazole-carboxamide, which forms methyl adducts at O6-position of guanine in DNA. The formation of O6-methylguanine then results in mismatch pairing with thymine during subsequent cycles of DNA replication, followed by DNA strand-break formation and eventually cell death (2). Critical to its effectiveness as an antitumor agent is the cellular expression of O6-methylguanine-DNA-methyltransferase (MGMT; refs. 3, 4), which removes the O6-methyl adducts and, thus, acts to repair temozolomide-induced DNA damage (2, 3). In a recently published study by Stupp et al. (5), patients with primary glioblastoma multiforme (GBM) treated with temozolomide plus radiotherapy followed by 6 months of adjuvant temozolomide were found to have a significant survival advantage compared with those patients treated with radiotherapy alone. Further analyses indicated that the greatest survival benefit was obtained in patients whose tumors contained methylated MGMT, which presumably resulted in a suppression of MGMT expression and activity (6). These results have led to temozolomide plus radiotherapy being the newly accepted standard of care for GBMs. However, whereas this combined modality increased survival times, the 5-year survival for GBM remains dismal; thus, understanding the processes

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responsible for the temozolomide + irradiation effectiveness may lead to further improvements in GBM therapy.

In the GBM treatment protocol, temozolomide was delivered during radiotherapy (5, 7), which suggests that survival benefits provided by the combined modality may be the consequence of a temozolomide-mediated radiosensitization. However, initial studies using established GBM cell lines (8), which do not express MGMT, indicated that exposure to temozolomide for 24 to 96 h before irradiation has no effect on their in vitro (intrinsic) radiosensitivity. Temozolomide delivered after irradiation was also reported to have no effect on the radiosensitivity of glioma cell lines that do or do not express MGMT (9). In contrast, Chakravarti et al. (10) recently reported that temozolomide delivered 2 h before radiation significantly enhanced the radiosensitivity of GBM cell lines established from primary culture that do not express MGMT but not of GBM cell lines that do express MGMT. Whether the variations in treatment protocols or the different cell lines used account for the inconsistent conclusions regarding the status of temozolomide as a radiosensitizing agent is unclear. Therefore, to further investigate the effects of temozolomide on tumor cell radiosensitivity, we have extended the studies of Chakravarti et al. (10) to the established GBM cell line U251. Moreover, because of the potential of combining temozolomide with radiation in the treatment of breast tumor metastases to the brain, we also evaluated a human breast tumor cell line that we have previously shown to be more radiosensitive, we have extended the studies of Chakravarti described (14). Slides were examined on a Leica DMRXA fluorescence microscope. Images were captured with a Photometrics Sensys CCD camera (Roper Scientific) and imported into IP Labs image analysis software package (Scanalytics, Inc.). For each treatment condition, γH2AX foci were determined in at least 150 cells. Cells were classified as positive (i.e., containing radiation-induced γH2AX foci) when more than five foci were detected.

Mitotic catastrophe. The presence of fragmented nuclei was used as the criteria for defining cells undergoing mitotic catastrophe. To visualize nuclear fragmentation, cells were fixed with methanol for 15 min at -20°C, stained with rabbit anti–α-tubulin antibody (Sigma-Aldrich) followed by staining with Texas red–conjugated secondary antibody (Jackson Immunomou Research Laboratories, Inc.). Nuclei were counterstained with 4’,6-diamidino-2-phenylindole. A single field containing 300 cells was selected at random for each treatment and photographed with epi- fluorescent. Nuclear fragmentation was defined as the presence of two or more distinct nuclear lobes within a single cell.

In vivo tumor model. Four to six-week-old female nude mice (Fredrick Labs) were used in these studies. Mice were caged in groups of five or less, and all animals were fed a diet of animal chow and water ad libitum. Tumor cells (5 × 10^6 cells) were injected s.c. into the right hind leg. Irradiation was performed using a Pantak irradiator with an absorbed dose of 4 Gy. To obtain tumor growth curves, perpendicular diameters of the tumors were measured every 2 days with digital calipers, and volumes were calculated using formula (L × W × W)/2. Tumors were followed until the tumors of the group reached a mean size of 2,000 mm³. Specific tumor growth delay was calculated for each individual animal in a treatment group as the number of days for the mean of the treated tumors to grow to 2,000 mm³ minus the number of days for the mean of the control group to reach the same size. SEs in days were calculated about the mean of the treated groups. Each experimental group contained 6 mice; 10 mice were included in the control group. All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals.

Statistical analysis. In vitro experiments were repeated thrice and statistical analysis was done using a Student’s t test. Data are presented as mean ± SD. A probability level of a P value of <0.05 was considered significant.

**Materials and Methods**

**Cell lines and treatment.** The U251 human GBM cell line was obtained from the National Cancer Institute, Frederick Tumor Repository. The breast tumor brain metastatic cell line MDA-MB-231BR (11) was supplied by the laboratory of Patricia Steeg (National Cancer Institute, Bethesda, MD). Cells were grown in DMEM (Invitrogen) with glutamate (5 mmol/L) and 10% fetal bovine serum and maintained at 37°C, 5% CO₂. Temozolomide, provided by the Developmental Therapeutics Program of the National Cancer Institute, was reconstituted in DMSO (100 mmol/L) and stored at -20°C. Cultures were irradiated using a Pantak X-ray source at a dose rate of 2.28 Gy/min.

**Clonogenic assay.** Cultures were trypsinized to generate a single-cell suspension and a specified number of cells were seeded into each well of a six-well tissue culture plate. After allowing cells time to attach (4 h), cultures received temozolomide (50 µmol/L for U251 and 25 µmol/L for MDA-MB-231BR) or DMSO (vehicle control) for 1 h before irradiation: medium was then removed and replaced with drug-free medium. Ten to fourteen days after seeding, colonies were stained with crystal violet, the number of colonies containing at least 50 cells was determined, and surviving fractions were calculated. Survival curves were then generated after normalizing for the amount of temozolomide-induced cell death. Data presented are the mean ± SE from at least three independent experiments.

**Cell cycle analysis.** Evaluation of cell cycle phase distribution was performed using flow cytometry. The treatment protocols were essentially the same as in the clonogenic survival experiments, except that the cells were initially seeded into 10-cm dishes. Samples were fixed, stained with propidium iodide, and analyzed using flow cytometry (Guava Technologies). To evaluate the activation of G₂ cell cycle checkpoint, mitotic cells were distinguished from G₂ cells, and the mitotic index was determined according to the expression of γH2AX (Upstate Biotechnology) as detected in the 4N DNA content population by the flow cytometric method of Xu et al. (13). In this assay, loss of mitotic cells (reduced mitotic index) reflects the onset of G₂ arrest.

**Apopotic cell death.** The Guava Nexin assay (Part Number 4500-0161) was performed following the manufacturer’s instructions. Briefly, 3.0 × 10⁶ cells (50 µL) were added to a 150 µL staining solution containing 135 µL 1× apoptosis buffer, 10 µL Annexin V-PE, and 5 µL of 7-AAD. The cells were incubated in the dark at room temperature for 20 min. Samples (2,000 cells per well) were then acquired on the Guava EasyCyte system.

**Immunofluorescent staining for γH2AX.** Immunofluorescent staining and quantifying of γH2AX nuclear foci was performed as previously described (14). Slides were examined on a Leica DMRXA fluorescent microscope. Images were captured with a Photometrics Sensys CCD camera (Roper Scientific) and imported into IP Labs image analysis software package (Scanalytics, Inc.). For each treatment condition, γH2AX foci were determined in at least 150 cells. Cells were classified as positive (i.e., containing radiation-induced γH2AX foci) when more than five foci were detected.

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Results

To determine the effects of temozolomide on GBM tumor cell radiosensitivity, clonogenic survival analysis was performed on the U251 cell line. In these studies, we used a temozolomide concentration of 50 μmol/L, which corresponds to the serum concentration achieved in humans using the standard treatment protocol of 150 mg/m²/d (15). A 1-h exposure to temozolomide (50 μmol/L) resulted in a surviving fraction of ~40% (see Fig. 1), which is in the appropriate range for evaluating clonogenic survival in combination with radiation. For the combination protocol, 1 h after temozolomide addition, U251 cells were irradiated followed by a change to drug-free medium with colony-forming efficiency determined 10 days later. As shown in Fig. 1A, this temozolomide pretreatment increased U251 radiosensitivity with a dose enhancement factor at a surviving fraction of 0.10 of 1.32. To determine whether this radiosensitization was unique to the U251 GBM cell line, studies were extended to the breast tumor brain metastasis cell line MDA-MB-231BR cells. Because MDA-MB-231BR cells were more sensitive to temozolomide-induced cytotoxicity than U251, a drug concentration of 25 μmol/L was used, which resulted in a surviving fraction of 43%. As shown in Fig. 1B, a 1-h pretreatment with temozolomide enhanced MDA-MB-231BR cell radiosensitivity with a dose enhancement factor at a surviving fraction of 0.10 of 1.30.

To investigate the cellular processes through which temozolomide enhances radiosensitivity, we focused on the U251 cell line. With respect to defining the mechanisms of temozolomide-induced radiosensitization, an advantage to using the relatively short preexposure period of 1 h is that it reduces the likelihood of cells redistributing into a radiosensitive phase of the cell cycle. This was verified using flow cytometry of propidium iodide stained cells: After 1 h of exposure to temozolomide (50 μmol/L), there was no change in the distribution of U251 cells across the cell cycle (data not shown).

Another potential source of radiosensitization is the abrogation of the G2 checkpoint, which is considered to protect against radiation-induced cell death (16). The method of Xu et al. (13) was used to define the effects of temozolomide on the radiation-induced activation of the G2 checkpoint. This assay determines the percentage of mitotic cells in the 4N DNA content population according to the flow cytometric analysis of γH2AX, which is specifically expressed in mitotic cells. Performed as a function of time after irradiation, this analysis provides a measure of the progression of G2 cells into M phase and, thus, the activation of the G2 checkpoint. As shown in Fig. 2, irradiation (2 Gy) resulted in a reduction in the mitotic index by 1 h, reaching a maximum decrease at 3 h, indicative of activation of the G2 checkpoint. Temozolomide treatment only had no effect on the mitotic index nor did it affect the reduction induced by radiation. These results indicate that temozolomide-induced radiosensitization does not involve abrogation of the G2 checkpoint.
Chakravarti et al. (10) reported that the temozolomide-mediated radiosensitization of primary GBM cell lines involved an enhancement in radiation-induced apoptosis. To define the contribution of apoptosis to the temozolomide-mediated radiosensitization of U251 cells, Annexin staining was determined in cells at 24 and 72 h after irradiation (2 Gy). As expected for a solid tumor cell line, radiation induced little apoptotic cell death; essentially the same level of apoptosis was detected in U251 cultures treated with temozolomide only. The combination protocol shown to enhance radiation-induced death in Fig. 1 had no effect on the frequency of apoptotic cell death (Fig. 3). These data indicate that the temozolomide-mediated radiosensitization of U251 glioma cells does not involve enhanced susceptibility to apoptosis.

A critical determinant of radiation-induced cytotoxicity is the induction and repair of DNA damage, specifically DSBs. As a measure of radiation-induced DNA damage, we evaluated induction of nuclear foci of γH2AX, which has been established as a sensitive indicator of DNA DSBs with the dispersion of foci corresponding to DSB repair (17). U251 cells were exposed to temozolomide for 1 h and, as in the cell survival experiments, irradiated (2 Gy), fed with temozolomide-free medium, and γH2AX foci determined at times out to 24 h. Exposure of cells to temozolomide only for 1 h resulted in a significant increase in the number of γH2AX foci, a level that steadily declined out to at least 24 h after drug removal (Fig. 4). Irradiation (2 Gy) induced a significant increase in the number of γH2AX foci as detected at 1 h, which progressively declined out to 24 h. Exposure to temozolomide followed by 2 Gy resulted in a greater number of γH2AX foci than either of the individual treatments at 1 and 6 h, although the increase above the individual treatments was less than additive. However, the number γH2AX foci at 24 h was greater than additive in temozolomide + irradiation (21.90 ± 2.14 per cell) compared with the number of foci in cells treated with irradiation or temozolomide alone (8.43 ± 1.40 and 7.97 ± 1.22 per cell, respectively). The elevated level of γH2AX expression in cells 24 h after receiving temozolomide + irradiation combination suggests an inhibition of DNA DSB repair.

The inhibition of DSB repair by temozolomide, along with its failure to enhance radiation-induced apoptosis, suggested that temozolomide-induced radiosensitization involves an enhancement in mitotic catastrophe. To test this hypothesis, mitotic catastrophe was determined according to the number of cells with abnormal nuclei as a function of time after irradiation (18). As shown in the representative photomicrograph in Fig. 5A, cells undergoing mitotic catastrophe could be clearly distinguished after the individual treatment of irradiation (2 Gy) and temozolomide (1 h; 50 μmol/L) as well as the combination. As shown in Fig. 5B, there was a time dependent increase in the number of cells undergoing mitotic catastrophe after the individual treatments with radiation and temozolomide up to at least 72 h. In cells receiving the combination treatment, a significantly greater number of cells undergoing mitotic catastrophe were detected at 48 and 72 h posttreatment. These data thus suggest that the temozolomide-mediated radiosensitization is mediated by an inhibition of DSB repair, resulting in an increase in the cells undergoing mitotic catastrophe.

To determine whether the enhancement of tumor cell radiosensitivity measured in vivo could be translated into an in vitro tumor model, a tumor growth delay assay using U251 cells grown s.c. in the hind leg of nude mice was used. Mice bearing s.c. xenografts (172 mm³) were randomized into four groups: vehicle; temozolomide only (10 mg/kg); irradiation (4 Gy) only; and temozolomide (10 mg/kg) given by p.o. gavage 1 h before irradiation (4 Gy). Treatment was on the day of randomization. The tumor model, a tumor growth delay assay using U251 cells grown s.c. in the hind leg of nude mice was used. Mice bearing s.c. xenografts (172 mm³) were randomized into four groups: vehicle; temozolomide only (10 mg/kg); irradiation (4 Gy) only; and temozolomide (10 mg/kg) given by p.o. gavage 1 h before irradiation (4 Gy). Treatment was on the day of randomization. The growth rates for the U251 tumors exposed to each treatment are shown in Fig. 6. For each group, the time to grow from 172 mm³ (volume at the time of treatment) to 2,000 mm³ was calculated using the tumor volumes from the individual mice in each group (mean ± SE). The time required for tumors to grow from 172 to 2,000 mm³ increased from 18.6 ± 0.8 days for vehicle treated mice to 28.3 ± 1.6 days for temozolomide-treated mice. Irradiation treatment alone increased the time to reach 2,000 mm³ to 30.2 ± 1.6 days. However, in mice that received the temozolomide + irradiation combination, the time for tumors to grow to 2,000 mm³ increased to 62.3 ± 1.2 days. The absolute growth delays (the time in days for tumors in treated mice to grow from 172 to 2,000 mm³ minus the time in days for tumors to reach the same size in vehicle treated mice) were 9.7 ± 0.6 for temozolomide alone and 11.6 ± 0.6 for irradiation alone; the tumor growth delay induced by the temozolomide + irradiation treatment was 43.7 ± 0.4. Thus, the growth delay after the combined treatment was more than the sum of the growth delays caused by individual treatments. To obtain a dose enhancement factor comparing the tumor radioreponse in mice with and without temozolomide treatment, the normalized tumor growth delays were calculated, which accounts for the contribution of temozolomide to tumor growth delay induced by the combination treatment. Normalized tumor growth delay was defined as the time in days for tumors to grow from 172 to 2,000 mm³ in mice exposed to the combined modality minus the time in days for tumors to grow from 172 to 2,000 mm³ in mice treated with temozolomide only. The dose enhancement factor, obtained by dividing the normalized tumor growth delay in mice treated with temozolomide +
irradiation by the absolute growth delay in mice treated with radiation only, was 2.8. These data indicate that temozolomide significantly enhances the radiation-induced tumor growth delay of U251 xenografts.

Discussion

Clinical studies indicating that temozolomide delivered during radiotherapy provides a survival advantage in GBM treatment suggested that this DNA methylating agent may act to enhance the radiosensitivity of GBM cells. However, there exists conflicting preclinical data regarding the putative radiosensitizing activity of temozolomide. Using prolonged preirradiation exposures of 24 to 96 h to 5 μmol/L temozolomide, van Rijn et al. (8) reported that the effects of temozolomide plus radiation were additive in terms of cell killing for the D384 GBM cell line. These results were consistent with the essentially additive effects determined by isobologram analysis of the

Fig. 4. Influence of temozolomide on radiation-induced γH2AX foci. U251 cells growing in chamber slides were exposed to temozolomide (50 μmol/L) for 1 h, irradiated (2 Gy), fed fresh medium, and fixed at the specified times for immunocytochemical analysis for nuclear γH2AX foci. Foci were evaluated in 50 nuclei per treatment per experiment. A, representative micrograph images obtained from control cells and cells that had received temozolomide (50 μmol/L; 1 h) alone, irradiation (2 Gy) alone, and temozolomide (50 μmol/L; 1 h before radiation) plus irradiation at 24 h after 2 Gy radiation. B, vehicle-treated cells (empty columns), cells treated with temozolomide alone (hatched columns), and cells treated with the combination of temozolomide and irradiation (filled columns, 2 Gy). Columns, mean; bars, SE. Cells with more than five foci per nucleus were classified as positive for radiation-induced γH2AX. * P < 0.01 according to Student’s t test (irradiation versus temozolomide + irradiation).
temozolomide + irradiation combination in the U373 GBM cell line (19). In contrast, the studies described here showed that temozolomide induced a significant radiosensitization of U251 cells as well as the breast tumor brain metastasis cell line MDA-MB231BR. These different results may be explained by the temozolomide concentration used in the two studies. In our studies, tumor cells were exposed to 25 to 50 μmol/L, a clinically relevant concentration (15), for 1 h compared with the 5 μmol/L temozolomide for 24 to 96 h used in the previous study (8). The prolonged preirradiation exposure to temozolomide could redistribute cells through the cell cycle and/or induce a variety of stress responses; thus introducing a number of variables that could complicate the interpretation of results from cell survival experiments. As shown here, the exposure of...
U251 cells to 50 μmol/L temozolomide for only 1 h did not redistribute cells through the cell cycle phases before irradiation and did not induce excessive cytotoxicity, allowing for an accurate assessment of the effects of temozolomide on radiosensitivity. Chakravarti et al. (10) reported that exposure of GBM primary cell cultures (MGMT negative) to 100 μmol/L temozolomide for 2 h also resulted in radiosensitization, although the cytotoxicity induced by temozolomide alone in the two primary cultures was unclear in this study. Thus, suggest that the acute exposure to clinically relevant concentrations of temozolomide results in tumor cell radiosensitization, at least for cells that do not express MGMT.

As an initial investigation into the mechanism mediating the temozolomide-induced radiosensitization of U251 cells, we surveyed cellular processes known to be determinants of radiosensitivity. These studies indicated that temozolomide exposure did not result in cell redistribution into a radiosensitive phase of the cell cycle nor did it affect the activation of the G2 cell cycle checkpoint. In addition, the increased susceptibility to apoptotic cell death did not account for the temozolomide-induced radiosensitization. These apoptosis results are in contrast to those reported for the MGMT negative primary GBM cultures in which the temozolomide-induced radiosensitization was attributed to an increase in apoptosis (10). This discrepancy may be attributed to cell type specificity or the higher temozolomide concentration (100 μmol/L) used to treat the primary GBM cultures. As an alternative to apoptosis, irradiation can induce cell death via mitotic catastrophe. As shown here, temozolomide exposure of U251 cells resulted in a significant increase in radiation-induced mitotic catastrophe, which is consistent with the putative inhibition of DSB repair as described below.

A critical event in determining radiosensitivy is the repair of DNA DSB. Over the last several years, γH2AX expression has been established as a sensitive indicator of DSBs induced by clinically relevant doses of ionizing radiation (17, 20–22). At sites of radiation-induced DNA DSBs, the histone H2AX becomes rapidly phosphorylated (γH2AX) forming readily visible nuclear foci (17, 20). Although the specific role of γH2AX in the repair of DSBs has not been defined, recent reports indicate the dephosphorylation of γH2AX and dispersal of γH2AX foci in irradiated cells correlates with the repair of DNA DSBs (21, 22). Moreover, Macphail et al. (23), in their study of 10 cell lines, reported that the loss of γH2AX correlates with clonogenic survival after irradiation. The results presented here, showing that the expression of γH2AX in cells treated with the temozolomide + irradiation combination was significantly greater at 24 h after irradiation than that of the individual treatments, is thus suggestive of an inhibition of DSB repair. However, because temozolomide also induced a significant increase in γH2AX that also declined over the 24-h postirradiation period, it is not possible to conclude whether temozolomide inhibited the repair of radiation-induced DSBs or radiation inhibited the repair of temozolomide-induced DNA damage.

The data presented here indicate that temozolomide enhances the radiosensitivity of a tumor cell in vitro and, moreover, for the first time clearly show that temozolomide enhances the in vivo radiosensitivity of a GBM xenograft. In addition, the radiosensitivity of a cell line corresponding to a breast tumor brain metastasis was also enhanced by temozolomide, which suggests that combining temozolomide with whole-brain radiation therapy may be of benefit in brain metastasis therapy. The studies described here only addressed the effects of temozolomide on the radiosensitivity of tumor cells that do not express MGMT (6, 10, 19). For primary malignant cell lines that express MGMT, an acute preexposure to temozolomide was reported not to influence radiation-induced cell death (19). Published reports suggest that patients whose tumors express undetectable levels of MGMT benefit the most from the temozolomide plus radiotherapy combination (6, 10). The data presented here and as reported by Chakravarti et al. (10), suggest that this advantage is a consequence of temozolomide-induced radiosensitization. The putative radiosensitizing action of temozolomide leading to an improved survival of GBM patients supports the further development and application of radiosensitizing agents for GBM therapy.

**References**