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Inhibition of DNA repair for sensitizing resistant glioma cells to temozolomide

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Object. Temozolomide (TMZ) is a DNA alkylating agent currently used as adjuvant treatment for anaplastic astrocytomas. Its use in managing glioblastoma multiforme has been halted because of the lack of therapeutic effects due to cell resistance. Note that O^6-alkylguanine–DNA alkyltranferase (AGT) is a DNA repair enzyme that limits the efficacy of TMZ. In this study the authors investigated the ability of O^6-benzylguanine (BG), an AGT inhibitor, to sensitize a glioblastoma cell line resistant to TMZ.

Methods. The effects of TMZ alone (100 μg) and after exposure to BG (50 μg) were assessed in two glioblastoma cell lines, U373-MG and T98G, respectively, sensitive and resistant to TMZ. Cell viability was assessed using trypan blue; cell cycle analysis by fluorescence-activated cell sorter; and apoptosis and autophagy by terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) and acridine orange staining, respectively. Furthermore, the involvement of an autophagy marker, microtubule-associated light chain 3 (LC3), was assessed. Temozolomide suppressed the growth of and caused cell cycle arrest in the G_2–M phase of U373-MG cells but not T98G cells. Exposure to BG prior to TMZ resulted in a significant decrease in cell viability as well as cell cycle arrest in the G_2–M phase in T98G cells ($p < 0.05$). Although apoptosis was not detected on TUNEL staining, programmed cell death Type II (autophagy) was detected after exposure to BG and TMZ in T98G cells.

Conclusions. These results indicate that inhibition of AGT by BG can render previously resistant glioma cells sensitive to TMZ treatment. The mechanism of cell demise following BG-TMZ treatment seems to be autophagy and not apoptosis. Combination therapy involving TMZ and an AGT inhibitor may be an effective strategy to treat resistant gliomas.

Key Words • glioblastoma multiforme • anaplastic astrocytoma • autophagy • temozolomide

Malignant gliomas are the most prevalent type of brain tumor and are often refractory to even the most aggressive therapy.[2] With such treatment, including surgery, radiotherapy, and chemotherapy, the median survival time in a patient with GBM remains between 12 and 15 months.[8,19] Obviously, considering the devastating effects of this neoplasm and the limited success of currently available treatments, new therapeutic approaches are needed.

Temozolomide is a novel chemotherapeutic agent currently approved for use in treating anaplastic astrocytoma. It is an attractive drug because it can be administered orally, readily crosses the blood–brain barrier, and has minimal side effect.[23] This compound acts by methylating DNA at the N^7 and O^6 sites on guanine and the O^3 site on adenine. Alkylation of the O^6 site on guanine plays a critical role in the cytotoxicity of TMZ because it leads to the insertion of a thymine instead of a cytosine opposite to the methylguanine during subsequent DNA replication, thus leading to cell demise.[6]

A major obstacle in the treatment of malignant gliomas with the aid of TMZ is the resistance exhibited by GBM cells.[13,22] The major pathway of this resistance is thought to occur through the actions of the DNA repair protein AGT.[1] This protein acts by removing the alkyl adduct from the O^6 position of guanine. It is a so-called suicide enzyme because it is inactivated in the process of DNA repair and can only be replenished by de novo protein synthesis.[11,21] Note that BG is a potent inactivator of AGT and has been shown to potentiate the cytotoxic effects of alkylating agents in tumor cells.[7]

The aim of this study was to assess the effects of the AGT inhibitor BG to sensitize a GBM cell line resistant to TMZ.

Materials and Methods

Tumor Cell Lines

Human GBM cell lines A172, GB-1, T98G, U251-MG, U373-MG, and U87-MG were cultured as adherent cells in DMEM (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco-BRL), 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Reagents for Chemical Reactions

Temozolomide was kindly supplied by the Schering-Plow Re-

Abbreviations used in this paper: AGT = O^6-alkylguanine–DNA alkyltransferase; AVO = acidic vesicular organelle; BG = O^6-benzylguanine; cDNA = complementary DNA; DMEM = Dulbecco modified Eagle medium; FACS = fluorescence-activated cell sorter; GAPDH = glyceraldehyde-3-phosphate-dehydrogenase; GBM = glioblastoma multiforme; GFP = green fluorescent protein; LC3 = light chain constant region subgroup 3; mRNA = messenger RNA; RT-PCR = reverse transcription–polymerase chain reaction; TMZ = temozolomide; TUNEL = terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

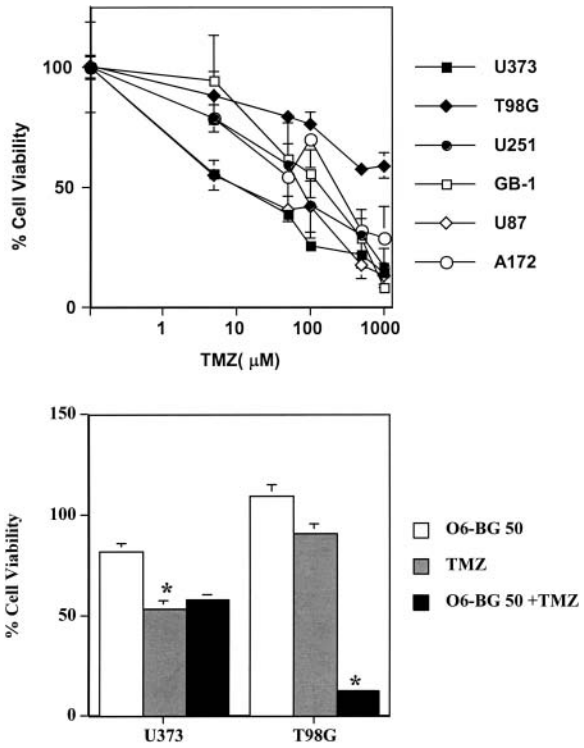

FIG. 1. *Upper:* Graph demonstrating effects of TMZ on the cell viability of malignant glioma cells. Tumor cells were seeded at 2×10^3 cells per well (0.1 ml) in 96-well flat-bottomed plates and incubated overnight at 37°C. After exposure to 100 mM TMZ for 3 days, cells cells were trypsinized and the number of cells was counted. The viability of untreated cells was regarded as 100%. Results shown are the means ± standard deviation of three independent experiments. *Lower:* Effects of TMZ and BG on cell viability in GBM cells. Bar histogram demonstrates the percentage of viable cells compared with baseline after exposure to 50 μg BG (O^6-BG 50), 100 μg TMZ, and 50 μg BG plus 100 μg TMZ (O^6-BG 50 + TMZ) for 72 hours. *$p < 0.5$ (unpaired Student t-test) compared to baseline. Data shown are representative of three experiments.

search Institute (Kenilworth, NJ). The BG was purchased from Sigma Chemical Co. (St. Louis, MO) and was dissolved in dimethyl sulfoxide (Sigma Chemical Co.). Acridine orange was obtained from Polysciences (Warrington, PA). The 3-methyladenine was also purchased from Sigma Chemical Co.

Cell Viability Assay

The cytotoxic effect of TMZ and the combination of TMZ and BG on malignant glioma cell lines was determined by performing a trypan blue dye exclusion assay, as described previously.[12] Tumor cells were seeded at 5×10^3 cells per well (0.1 ml) in 96-well flat-bottomed plates and incubated overnight at 37°C. Cells were exposed to BG (50 μM) in serum-free DMEM (total volume 50 μl) for 3 hours. The TMZ (100 μM) was then added to DMEM supplemented with 20% fetal bovine serum (total volume 50 μl). After exposure to TMZ for 3 days, the cells were trypsinized and the number of viable cells was counted.

Cell Cycle Analysis

For cell cycle analysis, untreated cells as well as tumor cells treated with TMZ (100 μM) with or without BG (50 μM) for 3 days were trypsinized, stained with propidium iodide by using a cellular DNA flow cytometric analysis reagent set (Boehringer Mannheim, Indianapolis, IN), and analyzed for DNA content by using the FACScan flow cytometer (Becton-Dickinson, San Jose, CA), as previously described.[16] Data were analyzed with the aid of CellQuest software (Becton-Dickinson). Dead cells were gated out by conducting pulse processing.

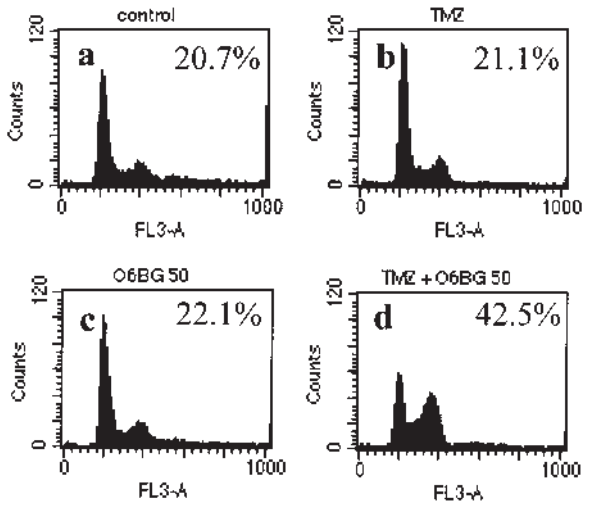

FIG. 2. Effects of TMZ and BG on the cell cycle of T98G GBM cells. Cell cycle analysis by FACS shows doubling of the number of cells in G_2–M arrest after exposure to TMZ and BG (d). This result was statistically significant: $p < 0.05$ (Student t-test) compared with control. Data shown are representative of three experiments. FL3-A = fluorescence channel 3 area for red fluorescence.

Apoptosis Detection Assay

Apoptosis was detected by performing a TUNEL assay with the ApopTag (fluorescein in situ apoptosis detection kit (Intergen, Purchase, NY). Briefly, digoxigenin-labeled nucleotides were added to the free 3′OH terminal resulting from DNA fragmentation associated with apoptosis by the enzyme terminal deoxynucleotidyl transferase. Fluorescein-linked antidigoxigenin antibody was added. We then analyzed the fluorescence by using the FACScan apparatus. Cisplatin was used as a positive control to induce apoptosis in glioma cell lines.

Detection and Quantification of AVOs With Acridine Orange

Autophagy is the process of sequestering cytoplasmic proteins into the lytic component and is characterized by the formation and promotion of AVOs. To detect and quantify the AVOs, we performed vital staining with acridine orange. In acridine orange–stained cells, the cytoplasm and nucleolus fluoresce bright green and dim red, whereas acidic compartments fluoresce bright red, as described previously.[7,25] The intensity of the red fluorescence is proportional to the degree of acidity and/or the volume of the cellular acidic compartment. Therefore, we could measure a change in the degree of acidity and/or the fractional volume of their cellular acidic compartment. Three days after exposure to TMZ, cells were stained with acridine orange at a final concentration of 1 μg/ml for a period of 15 minutes, removed from the plate with trypsin–ethylenediamine tetraacetic acid, and collected in phenol red–free growth medium. To inhibit autophagy, 2 mM 3-methyladenine was added after 1 day of treatment with TMZ. Green (510–530 nm) and red (> 650 nm) fluorescence emissions from 10^4 cells illuminated with a blue (488 nm) excitation light was measured using the FACScan flow cytometer and CellQuest software.

Transfection Experiments

The GFP-tagged rat LC3 expression vector was kindly provided by Dr. Mizushima (National Institute for Basic Biology, Okazaki, Japan) and transfected into T98G cells. Transfection was performed on 100-mm plates with 10 μg plasmid DNA per plate by using GenePorter (Gene Therapy System Inc., San Diego, CA) according

to manufacturer's instructions. After overnight exposure, cells were washed three times with phosphate-buffered saline and cultured in complete medium. Forty-eight hours after the medium change, cells were selected in medium containing 400 μg/ml of G-418 (Gibco-BRL). After 1 week of selection, G-418–resistant clones were randomly selected from the surviving colonies and used in the following experiments.

Fluorescence Microscopy

The GFP-LC3–transfected cells were cultured on chamber slide dishes (Fisher, Inc., Venice, FL). After treatment with TMZ with or without a 3-day exposure to BG, cells were fixed in 1% paraformaldehyde and were analyzed.

Reverse Transcription–PCR Analysis for LC3

The induction of LC3 mRNA in T98G cells was analyzed by semiquantitative RT-PCR amplification. Total RNA from T98G cells was isolated using a commercially available RNA isolation kit (Qiagen, La Jolla, CA). First-strand cDNA was synthesized with total RNA (0.5 μg) by using the Thermoscript RT-PCR kit (Invitrogen, La Jolla, CA). Two microliters of cDNA reaction was amplified using the Platinum Taq DNA polymerase PCR kit (Invitrogen) with primers LC3-5 (5′-ATGCCGTCGGAGAAGACCTT-3′) and LC3-3 (5′-TTACACTGACAATTTCATCCCG-3′).[9] The thermal cycle profile was 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute for 25 cycles. The amplified products were fractionated on a 2% agarose gel containing 0.5 μg/ml ethidium bromide. Gels were photographed using Polaroid film (type 667; Polaroid, Wayland, MA), and photographs were quantitatively scanned using Image software (National Institutes of Health, Bethesda, MD). The efficiency of cDNA synthesis from each sample was estimated by PCR with GAPDH-specific primers.

Statistical Analysis

Data were expressed as the means ± standard deviation. Statistical analysis was performed using the Student t-test (two-tailed). A statistical probability less than 0.05 was considered to be significant.

Results

Effect of TMZ and BG on Cell Viability

In a pilot study (Fig. 1 *upper*) we exposed six glioma cell lines to TMZ: U87-MG, A172, T98G, U373-MG, GB-1, and U251-MG. We determined that U373-MG was the most sensitive and T98G was the most resistant cell line to TMZ. Therefore, the experiments described in this study were conducted using these two cell lines.

To investigate the ability of BG to sensitize malignant glioma cell lines to TMZ cytotoxicity, we exposed U373-MG and T98G cell lines to 100 μM TMZ for 72 hours with or without a prior 3-hour exposure to 50 μM BG. After 72 hours we determined the percentage of viable cells compared with the untreated controls. The TMZ treatment alone significantly reduced cell viability in the U373-MG cell line ($p = 0.006$), but not in the T98G cells (Fig. 1 *lower*). The combination therapy of BG and TMZ did not increase the cytotoxic effect of TMZ in U373-MG cells, but the viability of T98G cells was significantly reduced compared with TMZ treatment alone ($p = 0.026$).

Effect of TMZ and BG on the Cell Cycle

To understand the mechanism(s) of action of BG, we performed DNA flow cytometric analysis. The TMZ treatment (100 μM, 72 hours) did not appreciably change the cell cycle profile of T98G cells. Exposure to BG (50 μM, 3 hours) prior to TMZ treatment markedly decreased the number of cells in the G_1 phase of the cell cycle and doubled the percentage of cells in the G_2–M phase (Fig. 2). These results indicated that BG is capable of sensitizing T98G cells to TMZ by inducing G_2–M arrest.

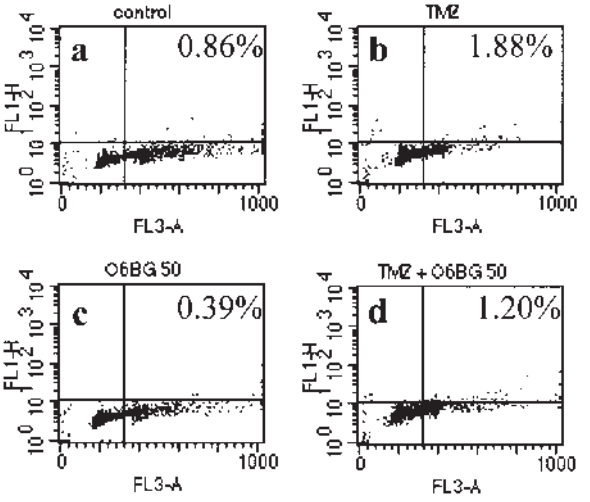

FIG. 3. Effects of TMZ and BG on apoptosis of T98G cells. The FACS analysis of TUNEL staining shows a lack of evidence of apoptosis after treatment with TMZ alone, BG alone, or BG-TMZ (d). Data shown are representative of three experiments. Similar results were found in U373-MG cells (data not shown). FL1-H = fluorescence channel 1 high for green fluorescence.

Programmed Cell Death After BG-TMZ Treatment of Malignant Glioma Cells

To ascertain whether treatment with BG-TMZ would result in programmed cell death Type I (apoptosis) or Type II (autophagy), we performed TUNEL and acridine orange staining. Apoptotic cells were not detected in T98G and U373-MG cells treated with TMZ alone (1.88% apoptotic cells) or with the combination of BG and TMZ (1.2%; Fig. 3). Cisplatin, used as a positive control to induce apoptosis, did in fact cause apoptosis in 64% of U373-MG cells. These results indicated that the cytotoxic effect of the combined BG-TMZ therapy is not due to the induction of apoptosis.

Acridine orange was used to detect AVO formation occurring during autophagy. It crosses membranes in its uncharged form, and in acidic compartments, its protonated form accumulates and fluoresces bright red. We performed FACS analysis to detect the amount of red fluorescence. In the TMZ-sensitive U373-MG cell line, the number of cells positive for bright red fluorescence rose from 6.3% in untreated cells to 28.1% in cells treated with TMZ (100 μM, 72 hours; Fig 4a–c). The U373-MG cells exposed to BG-TMZ failed to exhibit an increased number of cells positive for red fluorescence compared with cells exposed to TMZ treatment alone. The T98G cells treated with TMZ showed a small increase in the amount of red fluorescence–positive cells, rising from 9.2% in untreated cells to 13.7% in those treated with TMZ (Fig. 4d–g). On the other hand, the addition of BG to the TMZ treatment markedly increased the number of cells positive for red fluorescence to 24.9%. An inhibitor of autophagy, 3-methyladenine suppressed the formation of AVO in T98G cells treated with BG-TMZ (Fig.

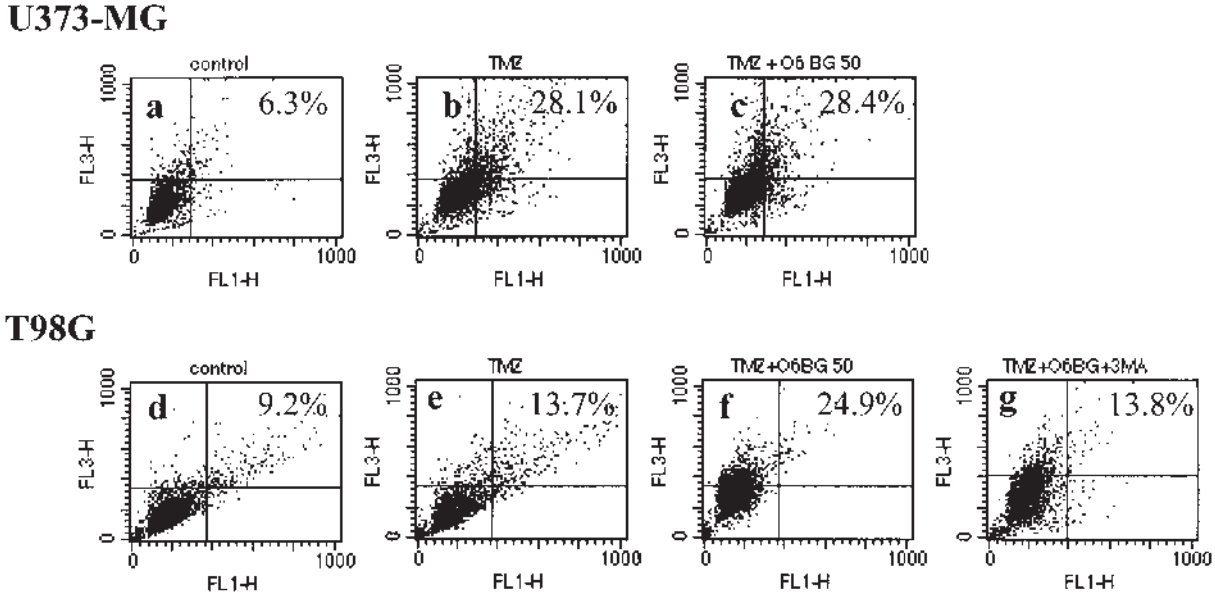

FIG. 4. Effects of TMZ and BG on autophagy on two GBM cell lines. The FACS analysis of acridine orange staining in U373-MG (a–c) and T98G (d–g) cells at baseline (control) and after treatment with TMZ or BG-TMZ. A marked increase in acridine orange–positive cells occurred after treatment with BG-TMZ in the TMZ-resistant T98G cells. These results corroborate that BG-TMZ causes cell demise by autophagy (see text). The autophagy inhibitor 3-methyladenine (3MA) reduces T98G cell's sensitivity to TMZ (g). Data shown are representative of three experiments.

4g). These results indicated that the increase in red fluorescence is due to the induction of AVO formation by BG-TMZ. From this, one could infer that the mechanism of cell death following BG-TMZ treatment is due to autophagy. The addition of BG sensitized the resistant T98G cells to AVO formation following exposure to TMZ.

Localization of the Microtubule Protein LC3 in T98G Cells Treated With BG-TMZ

The rat microtubule-associated protein–1 LC3 is associated with the autophagosome membrane. To detect the formation of autophagosomes following treatment with BG-TMZ, we established a line of T98G cells transfected with LC3 fused to GFP. With the aid of fluorescence microscopy, GFP-LC3–transfected cells exhibited diffuse green fluorescence with a few areas of a more punctate pattern indicating autophagosomes (Fig. 5a). Treatment with TMZ (100 μM, 72 hours) or BG alone did not change the pattern of green fluorescence from its baseline appearance (Fig. 5b and c). On the other hand, after exposure to both BG and TMZ there was a marked increase in the number of autophagosomes (Fig. 5d). These results corroborate the finding that BG-TMZ treatment promotes autophagy and induces autophagosome formation with concomitant LC3 localization in T98G cells.

The BG-TMZ treatment increases the expression of LC3 mRNA in T98G cells. The LC3 levels have been shown to correlate with the number of autophagosomes. Treatment of T98G cells with a combination of BG and TMZ induced AVO formation and the aggregation of GFP-LC3. To ascertain whether this treatment could increase the amount of LC3 in T98G cells, we performed semiquantitative RT-PCR analysis with primers for LC3. The LC3 mRNA is constitutively expressed in small amounts in T98G cells (Fig. 6). Following treatment with TMZ (100 μM, 72 hours), LC3 mRNA expression increased by 40%. In cells that had been exposed to BG (50 μM, 3 hours) prior to TMZ treatment, LC3 mRNA expression was enhanced by more than 60%. In contrast, cells exposed to BG-TMZ and 3-methyladenine had a drastically smaller increase in LC3 mRNA expression. Similar results were found in U373-MG cells (data not shown). These results confirmed that treatment with BG-TMZ increases the expression of LC3 mRNA in GBM cells.

Discussion

In this study we reported on the effects of exposure to BG prior to TMZ treatment in malignant T98G glioma cells resistant to TMZ. Exposure to BG prior to TMZ treatment resulted in a significant decrease in cell viability as well as cell cycle arrest in the G_2–M phase. Although apoptosis was not detected on TUNEL staining of T98G cells, autophagy was detected after exposure to BG and TMZ. Finally, the exposure of BG prior to TMZ treatment resulted in the increased expression of LC3 mRNA. Additional investigations of inhibitors of DNA repair must be pursued in vivo to corroborate these findings.

Inhibition of AGT Sensitizes Malignant Glioma Cells Otherwise Resistant to TMZ

Chemotherapeutic agents alkylating the O^6 position of guanine in DNA, such as carmustine, are used primarily to treat brain cancer, lymphoma, and gastrointestinal cancer.[7] Temozolomide is an alkylating agent that has demonstrated clinical antitumor activity[17,18] and a relatively well tolerated safety profile in Phase I and II clinical trials.[28] Note that TMZ is rapidly absorbed after oral administration and

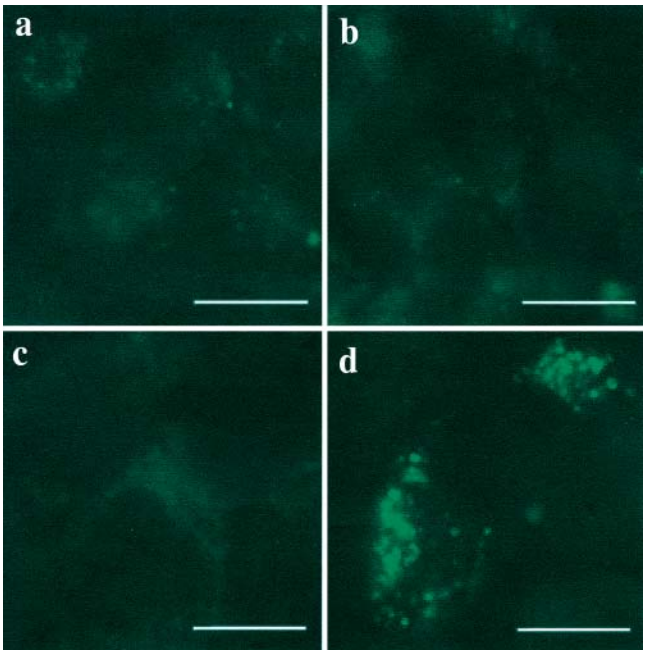

FIG. 5. Localization of the microtubule protein LC3 in T98G cells treated with BG and TMZ. Photomicrographs of fluorescent microscopy demonstrating T98G cells transfected with the microtubule LC3 protein fused to GFP. a: Baseline (control). b: After treatment with TMZ (100 μg). c: After treatment with BG (50 μg). d: After treatment with BG-TMZ. Treatment with TMZ or BG did not change the pattern of green fluorescence from its baseline appearance. In contrast, after exposure to BG-TMZ there was a marked increase in the number of autophagosomes.

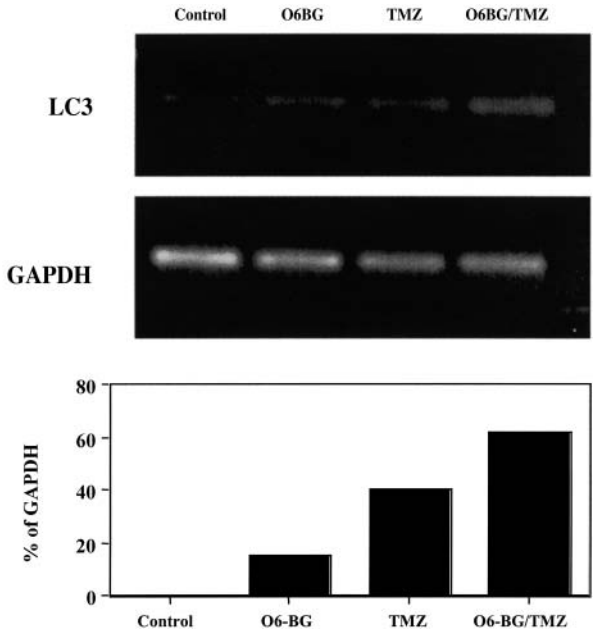

FIG. 6. The BG-TMZ treatment increases the expression of LC3 mRNA in T98G cells. *Upper:* Results of RT-PCR analysis of LC3 mRNA in T98G cells at baseline (control), after treatment with TMZ (100 μg), after treatment with BG (50 μg), and after BG-TMZ treatment. There was a marked increase in the expression of LC3 mRNA after treatment with BG-TMZ in T98G cells. *Lower:* Histograms of the semiquantitative analysis. Results are expressed normalized to GAPDH expression. Data shown are representative of three experiments.

undergoes spontaneous hydrolysis at physiological pH to its active metabolites 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC). The action of TMZ has been extensively studied, primarily in leukemia and lymphoma cells. In addition, MTIC produces O^6-methylguanine in DNA, which in turn misrepairs with thymidine, thus triggering futile DNA mismatch repair and ultimately cell death. The DNA repair protein AGT has been shown to protect cells from the toxic and mutagenic effects of alkylating agents by removing lesions from the O^6 position of guanine. Furthermore, BG is a potent inactivator of AGT. Recent data show that the use of BG in conjunction with an O^6-guanine alkylating agent resulted in an increase in the sensitivity of cells to the cytotoxic effects of chemotherapeutic alkylating agents in ovarian cancer,[5] melanoma,[14] and leukemia.[24] In a very recent study, Hirose, et al.,[9] showed that in the human GBM cell line SF767, delayed repletion of O^6-methylguanine–DNA transferase resulted in a failure to protect the cells from TMZ-induced cytotoxicity. Our data demonstrated that the use of BG potentiates the cytotoxic effects of TMZ in GBM cells otherwise resistant to it. These findings corroborate the concept that inhibition of AGT plays a key role in developing new strategies for malignant gliomas.

Autophagy occurs in malignant glioma cells after exposure to BG and TMZ.

Accumulating evidence indicates that programmed cell death is not confined to apoptosis.[3,4] Cells use different pathways for active self-destruction, as reflected by their different morphology. Condensation of cytoplasm and preservation of the organelles is prominent in apoptosis. Acidic vacuole formation is prominent in autophagy.[20,26] Recent data show that autophagy is characterized by degradation of Golgi apparatus, polyribosomes, and endoplasmic reticulum, which precedes nuclear destruction.[3,4] Although published data have revealed the action of TMZ in hematopoietic neoplasms, its action in glioma cells remains largely undefined. In addition, glioma cells have been shown to be relatively resistant to apoptosis induced by DNA damage.[13,27] In our study we showed that cell demise after exposure to TMZ in GBM cells may or may not occur. In cells resistant to TMZ, concomitant exposure to the AGT inhibitor BG is necessary to induce cell demise. When cell demise occurs, however, it does so by autophagy and not apoptosis. This was demonstrated by the increased number of AVOs shown in T98G glioma cells after exposure to BG and TMZ. At the same time, TUNEL staining remained negative. Thus, GBM cells undergo demise by autophagy following exposure to TMZ.

The LC3 is Associated With the Autophagy Process Induced by BG-TMZ Treatment

Autophagy is a degradative pathway that involves the sequestration of cytoplasmic portions and intracellular organelles in a membrane vacuole called the autophagosome. These vesicles fuse with lysosomes, and the sequestered material is degraded. Because of the complexity of the autophagic pathway and its inaccessibility to external probes, little is known about the molecular mechanisms that regulate autophagy in higher eukaryotic cells.[16] Recently, it has

been asserted that LC3 is required to allow membrane isolation during autophagy.[15] In addition, LC3 levels have been shown to correlate with autophagosome numbers.[10] Thus, increased LC3 mRNA expression is additional evidence that autophagy occurs. In our study, by using PCR we showed that malignant glioma cells exposed to BG-TMZ treatment expressed an increased amount of microtubule protein LC3 mRNA. These results corroborate the findings that the cytotoxic effects of BG-TMZ are caused by autophagy.

Conclusions

Our data confirm that in GBM cell lines there is an intratumoral variability in the sensitivity to TMZ. Glioblastoma multiforme cells not sensitive to TMZ can become sensitized to this drug by inhibiting AGT. In addition, we have shown that cell demise after exposure of TMZ alone or in combination with the AGT inhibitor BG occurs by autophagy and not apoptosis. These findings should be taken into consideration when developing new strategies for adjuvant treatment of recurrent GBMs.

Acknowledgments

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