Induction of the DNA repair gene \(O^6\)-methylguanine–DNA methyltransferase by dexamethasone in glioblastomas

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**Object.** The DNA repair enzyme \(O^6\)-methylguanine–DNA methyltransferase (MGMT) inhibits the cytotoxic effect of alkylating agents on tumor cells. The presence of two nonconsensus glucocorticoid-responsive elements in the human MGMT promoter region indicates the potential regulation of MGMT expression by glucocorticoid agents. This study was performed to elucidate whether dexamethasone affects the expression of MGMT in glioblastoma multiforme (GBM) cells, thereby limiting the benefit of chemotherapeutic alkylating agents.

**Methods.** Four GBM cell lines (A172, T98G, U138MG, and U87MG) were exposed to the alkylating agent 1-(4-aminomethyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) with or without dexamethasone. The expression levels of MGMT were correlated with the cytotoxic effects of ACNU in GBM cells. In the presence of ACNU alone, dexamethasone alone, and the combination of both agents, messenger RNA expression of MGMT was induced to varying degrees with the highest increases seen in the later conditions. This dexamethasone-dependent induction of the MGMT gene was even observed in U87MG cells in which the promoter is methylated, although the absolute expression of MGMT mRNA was the lowest in that cell line. The induction of MGMT by dexamethasone was associated with an increased resistance of these cells to ACNU.

**Conclusions.** These results indicate that dexamethasone-mediated upregulation of MGMT limits the efficiency of alkylating agents in the treatment of malignant gliomas.

**Key Words** • \(O^6\)-methylguanine–DNA methyltransferase • dexamethasone • gene regulation • glioblastoma multiforme

The current management of malignant gliomas includes cytoreductive surgery, radiotherapy, and several adjuvant chemotherapy protocols. Antitumor alkylating agents have been widely used alone or in combination with other drugs to treat malignant gliomas. These alkylating agents convert guanine into \(O^6\)-alkylguanine in the DNA, which leads to covalent cross-linking between the DNA strands, inhibition of cellular division, and apoptosis in the dividing cell. The \(O^6\)-alkylguanine can be removed by the DNA repair enzyme MGMT, which has been shown to be one of the factors that contribute to alkylating agent resistance in tumors. In addition, several investigators have reported that, in malignant brain tumors, MGMT expression is closely correlated with clinical and experimental data. The human MGMT gene consists of five exons spanning more than 170-kb pairs. Its 5′ regulatory promoter has been characterized for known regulatory sequences and has been shown to possess two GREs, from which we may infer a potential role for glucocorticoid regulation in the induction of MGMT.

Most patients with a malignant glioma receive glucocorticoid medications for the management of cerebral edema, tumor mass effect, and radiotherapy side effects. The steroid-induced improvement in cerebral edema and the decrease in contrast enhancement on computerized tomography scans reflect the stabilization of the blood–tumor barrier and the peritumoral BBB. It is therefore imperative to elucidate whether glucocorticoid hormones affect MGMT gene expression in GBM cells and potentially limit the benefits of chemotherapy with alkylating agents. In this paper we report the effects of glucocorticoid hormones on MGMT gene expression in GBM cell lines and show the desensitization to ACNU that is created.

**Materials and Methods**

**Cell Culture, Treatment With ACNU, and the Cell Viability Assay**

The human GBM cell lines A172, T98G, U138MG, and U87MG were purchased from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco modified Eagle medium or minimal essential medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL) and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO2 at 37°C. Tumor cells in the logarithmic growth phase were plated in Falcon 100-mm culture dishes at a density of 105 cells per dish. After confirmation of cell adhesion 6 hours later, the cells were cultured for 24 hours in serum-free medium with or without 10 μg/ml ACNU. The media in the dishes were then replaced by serum-containing media and the cells remained in culture for up to 120 hours. The cells were counted at 24-hour intervals by using the trypan blue dye–exclusion method to determine their survival rates. To analyze the effect of dexamethasone on the cytotoxicity of ACNU, cells were
also plated in the manner just described. After we had confirmed that
the cells had attached to the dish, dexamethasone (10 μM) was added
to the medium in test samples and distilled water was added to the
medium in control samples. The media were then replaced with se-
num-free media that contained ACNU and the cells were cultured for
24 hours. The serum-free media were replaced by serum-containing
media, and the cells were cultured for up to 120 hours. Cell viability
was compared between untreated and treated groups by using the
trypan blue dye–exclusion method.

**Isolation of DNA and MS-PCR**

The DNA was extracted by using a DNeasy Tissue Kit (Qui-
gen, Valencia, CA) according to the manufacturer’s instructions. The
methylated status of the CpG island of the MGMT gene was de-
termined by a chemical modification of unmethylated cytosine to
uracil. The MS-PCR was performed with primers specific to ei-
ther methylated or modified unmethylated DNA, as previously de-
described. One microgram of extracted DNA underwent bisulfite
modification by using a CpGenome DNA modification kit (Inter-
Gen, Burlington, MA), according to the manufacturer’s instructions.
For PCR amplification, the primer sequences used for the unmeth-
ylated reaction were 5’-CTTTGAGTCTGATGTGCT-3’ (sense) and 5’-
AAGTTTCTCCTTCCAAAACAAACCA-3’ (antisense). The primers used for the methylated
reaction were 5’-TTTGACCTTCTGGATGTTT3’ (sense) and 5’-
GCACTTCTCCAAAACCAAGCG-3’ (antisense). The MS-PCR was performed on 1 μl of bisulfite-treated DNA under the following conditions: 96°C for 10 minutes; 50 cycles of 96°C for 20
seconds, the annealing temperature 57°C for 20 seconds, and 72°C for 45 seconds; and a final extension of 4 minutes at 72°C. Enzymatically methylated human male genomic DNA (CpGenome Universal
Methylated DNA; InterGen) was used as a positive control for meth-
ylation status. Only in the U87MG cell line. The other three cell lines, T98G, A172, and U138MG, do not contain any methylated DNA at this locus. The DNA was extracted by using a DNeasy Tissue Kit (Qia-
gen, Valencia, CA) according to the manufacturer’s instructions. The
methylated status of the CpG island of the MGMT gene was de-
termined by a chemical modification of unmethylated cytosine to
uracil. The MS-PCR was performed with primers specific to ei-
ther methylated or modified unmethylated DNA, as previously de-
described. One microgram of extracted DNA underwent bisulfite
modification by using a CpGenome DNA modification kit (Inter-
Gen, Burlington, MA), according to the manufacturer’s instructions.
Western Blot Analysis

The cells were harvested to measure total cell lysates with RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 50 mM Tris-HCl, pH 7.5) that contained a pro-
tease inhibitor mixture (Roche Diagnostics GmbH, Mannheim, Ger-
many) and 1 mM NaF plus NaVO₄. The cell lysates were extracted
using Dounce homogenizers. The proteins were quantified by per-
forming the Bradford protein assay (Bio-Rad, Inc., Hercules, CA).
Forty micrograms of each protein was electrophoresed on a 10%
polyacrylamide gel, transferred to a polyvinylidene fluoride mem-
brane, blocked with 5% skim milk, and reacted with anti-MGMT
polyclonal antibody and antiaction antibody (both from Santa Cruz
Biotechnology, Santa Cruz, CA). The immunoblotted complex was
visualized with the aid of the ECL Western Blotting system (Amer-
sham Pharmacia Biotech). The intensities of the stained bands were
quantified by densitometry and Image software (version 1.63; Na-
tional Institutes of Health, Bethesda, MD) was used to estimate the
expression ratios of each sample.

**Results**

**Correlation of mRNA Expression of MGMT in Human GBM Cell Lines With ACNU Sensitivity**

We determined the mRNA expression levels of MGMT by using real-time quantitative RT-PCR in the four human
GBM cell lines U138MG, A172, T98G, and U87MG. To normalize the expression levels of the four cell lines, we chose to compare their expression levels with the basal
**MGMT** mRNA level of U138MG, the cell line whose ex-
pression of the gene is highest, thereby yielding relative percentages. To investigate the correlation between ACNU sensitivity and **MGMT** gene expression further, the human
GBM cell lines were cultured with ACNU for up to 120 hours, and cell viability was determined. The cytotoxic effects of ACNU, as measured using trypan blue dye, differed significantly among these cell lines. The U87MG cells were
the most sensitive to ACNU at 120 hours, with only 25% cell viability. In contrast, the U138MG cells were the most resistant; more than 50% of the cells survived ACNU treat-
ment (Table 1). As shown in Table 1, the expression levels of **MGMT** mRNA in the four cell lines were strongly correlated with the cell line’s resistance to ACNU. As such, U87MG cells had the lowest expression level of **MGMT** and were most sensitive to the cytotoxic effects of ACNU, whereas U138MG cells expressed the highest amount of **MGMT** and were most refractory to ACNU’s effects.
Dexamethasone induction of MGMT in GBM cell lines

**Fig. 2.** Graph demonstrating the induction of MGMT mRNA by dexamethasone in the U87MG, T98G, and U138MG cell lines. The expression levels of MGMT mRNA are represented as expression was increased 1.8-fold compared to baseline expression levels. The addition of dexamethasone for 24 hours resulted in a higher expression level of MGMT in the U138MG cell line. Although all four cell lines demonstrate a similar relative increase in MGMT expression in response to dexamethasone treatment, U138MG displayed a 2.4-fold increase, A172, 2.2-fold increase; T98G, 1.9-fold increase; and U87MG, 2.4-fold increase, their absolute expression levels vary 11-fold. The induction of MGMT mRNA is expressed as the mean ± standard deviation of the ratios dexamethasone-treated/control cell expression. Experiments were performed three times independently.

**Methylation Status of the MGMT Gene Promoter Region**

Methylation of the CpG islands in gene promoter regions is a major factor in gene regulation and is correlated with transcriptional silencing. To confirm the methylation status of the MGMT promoter, DNA from U87MG, A172, T98G, and U138MG cells was isolated and examined by performing MS-PCR. Interestingly, the methylated promoter DNA was only present in the U87MG cells, which had the lowest basal expression of MGMT and the highest sensitivity to ACNU (Fig. 1).

**Dexamethasone-Mediated Activation of MGMT Gene Transcription**

To examine the effect of dexamethasone on the expression of MGMT, U87MG, A172, T98G, and U138MG cells were treated with dexamethasone for 24 hours, after which MGMT mRNA levels were assayed using real-time quantitative PCR. In U138MG cells, the mRNA expression of MGMT increased more than twofold after 24 hours of exposure and this expression level was more than 11-fold higher than that found in U87MG cells. Although the basal expression levels of the other three cell lines, T98G, A172, and U87MG, were significantly less (1.6-, 5.8-, and 11-fold less, respectively) than that of the U138MG cells, all three cell lines displayed more than a twofold relative induction of MGMT in response to the 24-hour exposure to dexamethasone (Fig. 2).

To elucidate further the influences of dexamethasone and ACNU on MGMT mRNA induction and the subsequent cellular resistance to ACNU, we selected the U138MG and U87MG cell lines, the lines demonstrating the highest and lowest levels of MGMT expression, respectively. This was done for the sake of simplicity because MGMT expression was expected, ACNU alone induced MGMT expression; in both cell types MGMT expression was increased 1.8-fold compared with basal expression levels. The addition of dexamethasone to ACNU induced MGMT expression to a level significantly higher than that attained using either agent alone (Fig. 3A and B). A 2.6-fold induction was seen in U138MG cells and a 3.5-fold induction in U87MG cells. Although the relative induction of mRNA was similar in these two cell types, the absolute levels of expression were much higher in the U138MG cells (Fig. 3C).

Because mRNA levels are used as a surrogate marker for protein expression, we performed a Western blot analysis of the U138MG and U87MG cell lines to verify protein induction (Fig. 4). There was an obvious induction of MGMT protein in both cell lines following exposure to dexamethasone, but the absolute protein level was much higher in the U138MG cells, corroborating our mRNA results. We could not perform a similar Western blot analysis of cells treated with ACNU alone or in combination with dexamethasone because of the high percentage of dead cells.

**Dexamethasone-Induced MGMT Expression Levels Correlate With Desensitization to ACNU**

It was our hypothesis that increased MGMT expression would lead to higher MGMT activity and increased resistance to ACNU toxicity. To confirm this in the GBM cell lines, we again exposed U138MG and U87MG cells to ACNU alone or in combination with dexamethasone. The exposure to ACNU alone resulted in cell death rates of 47 and 77% in U138MG and U87MG cells, respectively, reflecting the significantly higher MGMT expression level of the U138MG cells. In cells that had been treated with both
ANCU and dexamethasone, there was a significant decrease in cell death in both cell lines. There was a 23% higher survival rate in U87MG cells and an 11% increase in cell survival in U138MG cells, demonstrating anti-ACNU activity in these cells (Fig. 5). Notably, the resistance of these cell lines to ACNU, both in the presence and absence of dexamethasone, reflects the differences in MGMT mRNA expression levels between the two cell lines.

Discussion

Because of their ability to permeate the BBB, “chloroethylating” agents such as ACNU, BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea, also known as carmustine), and CCNU (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, also known as lomustine), and methylating agents such as procarbazine and temozolomide are widely used in patients with malignant gliomas, as an adjuvant treatment to surgery and radiotherapy. These drugs have been associated with modest increases in response rates and survival times. Their clinical effectiveness is attributed, in part, to their relatively high yields of cytotoxic O6-alkylguanine adducts in DNA. On the other hand, MGMT mediates the resistance to alkylating agents by removing these cytotoxic alkyl adducts from the O6 atom of guanine.

Protein expression levels of MGMT differ according to cell type and are low in some tumors. In approximately 30% of gliomas, there is no detectable level of MGMT. Absence of this enzyme may increase the sensitivity of brain tumors to alkylating agents. This lack of MGMT expression is not commonly due to a deletion, mutation, or rearrangement of the gene; therefore, other causes for its deficiency may be involved. One of the main epigenetic phenomena that can silence gene transcription in humans is DNA methylation. Esteller, et al., have demonstrated that the inactivation of MGMT by promoter hypermethylation is a common event in primary human neoplasms. Recently, they indicated that methylation of the MGMT promoter in malignant gliomas is a useful predictor of tumor responsiveness to alkylating agents.

Recently, the molecular mechanism of MGMT gene upregulation has been reported. The 5’ region preceding exon 1 in the genomic DNA sequence of the MGMT gene has two nonconsensus GREs. The GREs were found to be functional by the results of a DNAse I footprinting assay, glucocorticoid-dependent luciferase reporter gene activation, and in vitro transcriptional assays. These functional GREs could be activated in the presence of dexamethasone, resulting in an increase in MGMT levels.

In this study, we have demonstrated that MGMT expression levels strongly correlate with the cytotoxic effects of ACNU in GBM cells. We demonstrated that dexamethasone upregulates both MGMT mRNA and protein levels and this upregulation correlates with the anti-ACNU activity in GBM cells seen with dexamethasone exposure. We found an approximately 2- to 2.5-fold increase in mRNA and protein levels after a 24-hour dexamethasone treatment alone and a higher induction of expression following a combined dexamethasone and ACNU treatment. Furthermore, we found an approximate 1.4- to twofold increase in the expression of MGMT mRNA in cells treated with ACNU alone. Although it is already known that alkylating agents promote the expression of MGMT, we more importantly demonstrated that GBM cells treated with ACNU and dexamethasone together express significantly higher amounts of MGMT mRNA compared with ACNU alone.

Fig. 4. Western blots of MGMT protein levels in U87MG and U138MG cells before and after a 24-hour exposure to dexamethasone. In dexamethasone-treated cells there is an increase in protein expression. Protein expression in the U138MG cells is significantly higher than that in U87MG cells, mirroring the mRNA levels in these cell lines (Fig. 3). The β-actin controls display equal protein loading.

Fig. 5. Graphs demonstrating dexamethasone-mediated protection of U87MG (A) and U138MG (B) cells from ACNU toxicity. Survival was measured by performing trypan blue dye-exclusion assay. The percentage of surviving cells was plotted against exposure time to either ACNU alone (diamonds) or ACNU plus dexamethasone (squares). There was a significant increase in the percentage of surviving cells with the concomitant dexamethasone treatment, with a 23% and an 11% increase for U87MG and U138MG cells, respectively. The percentage of survival is expressed as the mean ± standard deviation of three independent experiments.
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treatment alone. When exposed to both ACNU and dexamethasone, these cells not only expressed higher levels of MGMT, but were also more resistant to the toxic effects of ACNU. Dexamethasone exposure conferred an additional 11 to 23% rate of cell survival to cells treated with ACNU. This recapitulates the common human scenario in which patients harboring a malignant glioma receive dexamethasone both before and concomitant with radiation and chemotherapy. This may partially explain the poor response rates of gliomas to alkylating chemotherapy because glucocorticoid-mediated upregulation of MGMT may limit its therapeutic efficiency.

Dexamethasone is used clinically because it reduces vascular permeability and repairs the BBB, thus decreasing cerebral edema. Its action is thought to be largely secondary to its affects on prostaglandin biosynthesis. Dexamethasone inhibits the activity of phospholipase A2, the first step in leukotriene and prostaglandin synthesis. Similar changes in vascular and BBB permeability can be affected by inhibiting enzymes found further downstream in the biosynthetic pathway, such as COX-2. Recently, Portnow et al. demonstrated, in a rat brain tumor model, that selective COX-2 inhibitors may be as effective as dexamethasone in controlling peritumoral cerebral edema and in prolonging survival. Coupled with our results, this may indicate a possible role for selective COX-2 inhibitors in the treatment of mild cerebral edema in patients with glioma who are undergoing alkylating agent chemotherapy. Additional in vivo animal studies are required to determine more fully the role of dexamethasone in decreasing tumor sensitivity to alkylating agent chemotherapy.

Conclusions

We have demonstrated that dexamethasone-mediated upregulation of MGMT limits the therapeutic efficiency of the alkylating agent ACNU in GBM cells. We believe that additional laboratory investigations are required for a proper evaluation of the chemotherapeutic effects of dexamethasone as they relate to its desensitizing effect in the presence of alkylating agents used to treat malignant gliomas. Recent findings indicate that COX-2 inhibitors may be a viable alternative to dexamethasone for the treatment of cerebral edema.

References


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