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Carmustine-Resistant Cancer Cells Are Sensitized to Temozolomide As A Result of Enhanced Mismatch Repair during the Development of Carmustine Resistance[S]

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ABSTRACT

The cytotoxicity of the monofunctional alkylator temozolomide (TMZ) is mediated by mismatch repair (MMR) triggered by O^6-alkylguanine, whereas MMR protects cells against bifunctional alkylators, including carmustine (BCNU). Therefore, TMZ may be cytotoxic to BCNU-resistant cancer cells because MMR affects sensitivity to TMZ and BCNU in a converse way. We evaluated TMZ cytotoxicity on BCNU-resistant variant (CEM-R) compared with the parental CCRF-CEM cell line (CEM-S). The mechanisms of its BCNU-resistance involved DNA repairs including nucleotide excision repair, base excision repair, alkylguanine alkyltransferase, MMR, and apoptotic and survival pathways. In particular, transcript levels of MMR-related hMLH1 and hMSH2 were enhanced in CEM-R cells. CEM-R cells were 8-fold more BCNU-resistant but surprisingly 9-fold more TMZ-sensitive than were CEM-S cells. Although TMZ-induced adducts include *N*-alkylated purines and O^6-alkylguaine, DNA excision repair was enhanced in CEM-R cells, suggesting the efficient repair of *N*-alkylation adducts. Cotreatment with methoxyamine, a base excision repair inhibitor, did not sensitize CEM-R cells to TMZ, suggesting little or no contribution of *N*-alkylation to TMZ-induced cytotoxicity. Cotreatment with O^6-benzylguanine, an alkylguanine alkyltransferase inhibitor, further sensitized CEM-R cells to TMZ, confirming the cytotoxic impact of O^6-alkylguanine. Cotreatment with cadmium chloride, an MMR inhibitor, disrupted the sensitivity of CEM-R cells to TMZ. The sensitivity to TMZ was reversed in the CEM-R variant clone that had been established by transfecting CEM-R cells with short hairpin hRNA against hMLH1, suggesting the critical role of MMR on sensitization to TMZ. In conclusion, BCNU-resistant CEM-R cells were sensitized to TMZ as a result of enhanced MMR during the development of BCNU resistance.

Because the prognosis of most malignant brain tumors is poor despite extensive surgical resections and intensive radiotherapy, chemotherapy is another important modality to improve the clinical outcome (Fine et al., 1993; Stupp et al., 2006). One of the most widely used anticancer agents for brain tumors is a bifunctional alkylating nitrosourea, 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine, BCNU) (Fine et al., 1993; Stupp et al., 2006). BCNU has been somewhat effective, but its clinical activity is limited by tumor cell resistance (Drabløs et al., 2004). A monofunctinal alkylator, 3,4-dihydro-3-methyl-4-oxoimidazo [5,1-*d*]-*as*-tetrazine-8-carboxamide (temozolomide, TMZ), is a relatively new anticancer agent that has exhibited good single-agent activity in patients with brain tumors that had relapsed after treatment, including nitrosourea-based chemotherapy (Stupp et al., 2001; Mason and Cairncross, 2005). TMZ has thus provided better clinical outcome than BCNU, although no formal comparative trials have been performed.

The superiority of TMZ over BCNU, especially the efficacy of TMZ against BCNU-resistant tumors, however, has not yet been mechanistically elucidated. Both monofunctional and bifunctional alkylators create a variety of DNA adducts in cancer cells, such as monoadducts on N^7-alkylguanine, N^1-alkylguanine, N^3-alkyladenine, and O^6-alkylguanine, and diadducts within or between DNA strands. The cytotoxicity of monofunctional agents, including TMZ, is due mainly to alkylation of O^6-guanine and the subsequent mismatch repair (MMR) response (Fink et al., 1998; Mason and Cairn-

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ABBREVIATIONS: BCNU, carmustine or 1,3-bis(2-chloroethyl)-1-nitrosourea; TMZ, temozolomide or 3,4-dihydro-3-methyl-4-oxoimidazo[5,1-*d*]-*as*-tetrazine-8-carboxamide; MMR, mismatch repair; NER, nucleotide excision repair; BER, base excision repair; MGMT, O^6-methylguanine-DNA methyltransferase; CCNU, lomustine or 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; ACNU, nimustine or 1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride; MX, methoxyamine; XTT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate; RT-PCR, reverse transcription-polymerase chain reaction; GST, glutathione transferase; shRNA short hairpin RNA; APE, AP endonuclease; BG, O^6-benzylguanine.

cross, 2005). Interstrand cross-links formed through the intermediate production of O^6-alkylguanine are critically cytotoxic lesions for bifunctional agents, including BCNU (Wiencke and Wiemels, 1995). The cytotoxic effect of alkylating agents is limited by a number of factors including DNA repair (Chaney and Sancar, 1996; Panasci et al., 2001). The adducts formed by alkylating agents undergo DNA repair specific for the type of adduct. *N*-Alkylation products are repaired by nucleotide excision repair (NER) and base excision repair (BER) (Wiencke and Wiemels, 1995; Chaney and Sancar, 1996; Plosky et al., 2002; Drabløs et al., 2004). O^6-Alkylguanine is repaired primarily by O^6-alkylguanine-DNA alkyltransferase (MGMT; Esteller et al., 2000). Interstrand cross-links are repaired by NER and recombination (Chaney and Sancar, 1996; Panasci et al., 2001). Deficiency in MMR leads to cellular insensitivity to monofunctional alkylators (Fink et al., 1998), whereas MMR is involved in cross-link repair induced by bifunctional agents (Aquilina et al., 1998; Fiumicino et al., 2000; Pepponi et al., 2003). There is no complete overlap between monofunctional and bifunctional agents in these repair functions. Therefore, the efficacy of TMZ may be distinct from BCNU in the context of DNA repair as the mechanism of drug resistance.

MMR seems to be associated with the expression of cytotoxicity of both monofunctional and bifunctional agents. With monofunctional agents, when MGMT capacity is saturated by an excess of O^6-alkylguanine produced, O^6-alkylguanine-thymine mispairing subsequently occurs by the erroneous incorporation of thymine instead of cytosine opposite the O^6-alkylguanine during DNA replication. The MMR system recognizes this mismatch and incises and excises the thymine-containing strand, but thymine is again incorporated into the repaired strand opposite O^6-alkylguanine. This triggers repetitive but unsuccessful MMR, leading to continuous DNA strand breaks and consequently apoptosis (Drabløs et al., 2004). A proficient MMR is thus necessary for the expression of cytotoxicity by monofunctional agents including TMZ. Conversely, loss of MMR is related to the hypersensitivity of tumor cells to bifunctional alkylators such as BCNU and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine, CCNU) (Aquilina et al., 1998; Fiumicino et al., 2000; Pepponi et al., 2003). It has then been suggested that MMR is involved in the protection of cells against cross-linking insults (Reddy and Vasquez, 2005; Wu et al., 2005). MMR activity therefore seems to be inversely associated with the cytotoxicity of TMZ and BCNU.

We hypothesized that the cytotoxicity of monofunctional agents would be enhanced in cancer cells that have become resistant to bifunctional agents, because the cells might acquire increased MMR response through the development of drug resistance. In this study, we evaluated the cytotoxic effect of TMZ on cancer cells that had been cultured to be resistant to BCNU in vitro. The sensitivity to TMZ and BCNU was compared in association with various DNA repair pathways including DNA excision repairs, MGMT, and MMR.

Materials and Methods

Chemicals and Reagents. BCNU (Sigma, St. Louis, MO) and O^6-benzylguanine (BG) (Sigma) were dissolved in 99% ethanol immediately before use. TMZ (Schering-Plough K. K., Osaka, Japan) and 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (nimustine, ACNU) (Sigma) were dissolved in 100% dimethylsulfoxide immediately before use. Methoxyamine (MX) (Wako Pure Chemicals, Osaka, Japan) and cadmium chloride ($CdCl_2$) (Nacalai, Kyoto, Japan) were dissolved in sterilized water.

Cell Culture. Human leukemia CCRF-CEM cells (CEM-S), which naturally have a low level of MMR activity (Taverna et al., 2000; Matheson and Hall, 2003), and a BCNU-resistant variant CEM (CEM-R) were used. To develop CEM-R cells, CEM-S cells were treated with escalating concentrations of BCNU with half of the IC_{50} (0.7 μM) for CEM-S cells as the initial concentration. The cells underwent subsequent passages with gradually increasing concentrations of BCNU for 8 months. Consequently, the cells grew in the media with 10 μM BCNU, and then one cell line resistant to BCNU (CEM-R) was cloned by the limiting dilution method. CEM-R cells acquired 2-fold increase in DNA excision repair activity including NER (Yamauchi et al., 2003). Erythroleukemia cell line K562, pre-B acute lymphoblastic leukemia cell line CCRF-697, and two myeloma cell lines RPMI8226 and U266 were also used.

Drug Treatment. Cells were incubated with various concentrations of TMZ, BCNU, ACNU, MX, BG, and $CdCl_2$, alone or in combination, for the time periods indicated, followed by washing in fresh media and subsequent incubation.

Proliferation Assay. To evaluate the growth inhibitory effect of each agent, the sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay was performed according to the manufacturer's instructions (Roche, Indianapolis, IN) with slight modifications (Yamamoto et al.,

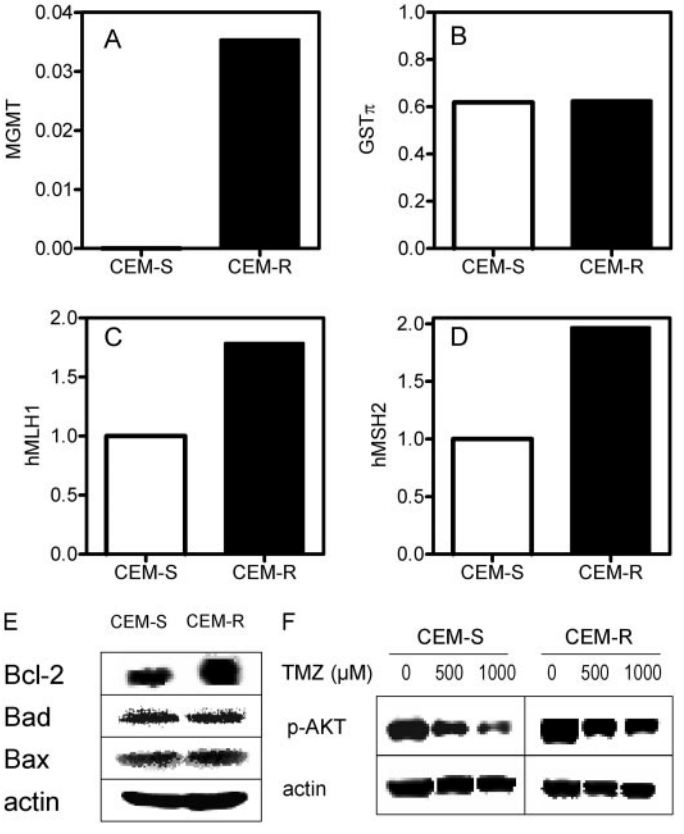

Fig. 1. A–D, transcript levels of repair-related enzymes including MGMT (A), GSTπ (B), hMLH1 (C), and hMSH2 (D) were determined using real-time RT-PCR. E, Western blot analyses of whole-cell lysates using antibodies against Bcl-2, Bad, and Bax. F, inhibition of phosphorylation of AKT. Cells (1×10^7 cells) were incubated for 6 h with or without TMZ at 500 or 1000 μM, followed by Western blotting using anti-phosphorylated AKT antibody.

2007). The doubling time for proliferation was also evaluated using the trypan blue dye exclusion assay (Yamamoto et al., 2007).

Quantitation of Apoptotic Cell Death. To evaluate cytotoxicity, apoptotic cell death was determined morphologically by staining nuclei of cells with Hoechst 33342 (Sigma) at 24 h after treatment (Yamauchi et al., 2002). Two hundred nuclei per treatment condition were then counted under UV illumination.

Alkaline Single-Cell Gel Electrophoresis (Comet) Assay. To evaluate the kinetics of TMZ-induced DNA excision repairs, the alkaline Comet assay was performed as described previously (Yamauchi et al., 2001, 2002, 2003). DNA single strand breaks were quantitated and expressed as the "tail moment."

Real-Time RT-PCR. To determine what factor might be related to drug sensitivity, the transcript levels of MGMT, glutathione transferase (GST) π, hMLH1, and hMSH2 were determined by real-time RT-PCR using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). The sequences of sense and antisense primers of GSTπ and MGMT were prepared by Mitsubishi Kagaku Bio-Clinical Laboratories (Tokyo, Japan), whereas the primers for hMLH1 and hMSH2 were purchased from Applied Biosystems (Supplemental Table 1). The absolute standard curve quantitation method was used for MGMT and GSTπ, and the relative standard curve quantitation method was used for hMLH1 and hMSH2.

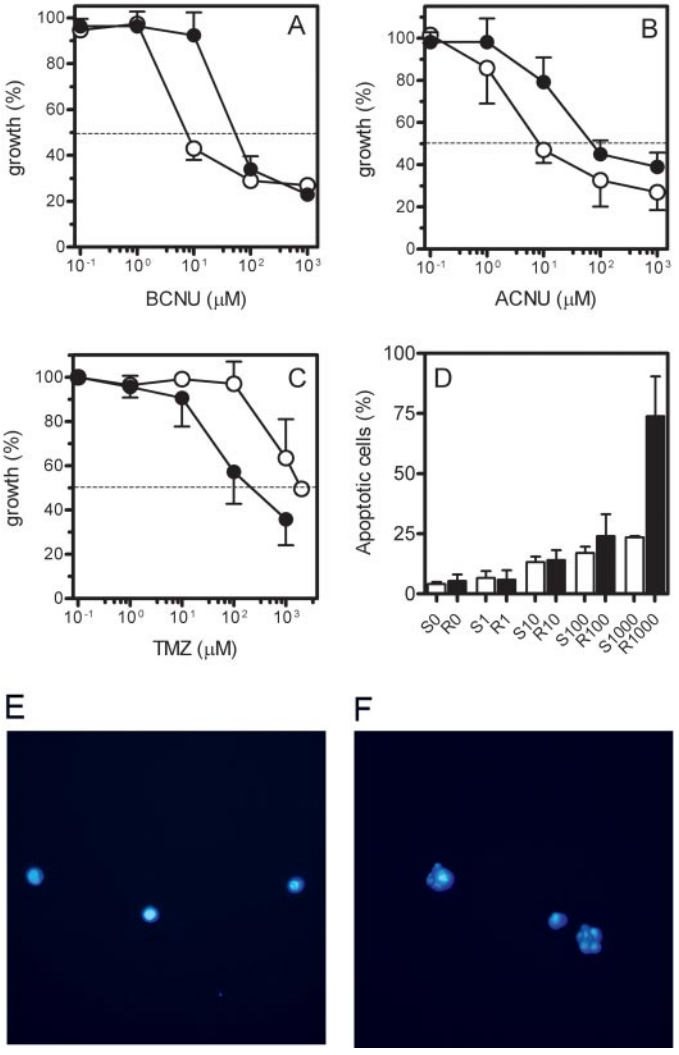

Fig. 2. A–C, growth inhibition curves of CEM-S (○) and CEM-R (●) cells. Cells were incubated for 72 h with various concentrations of BCNU (A), ACNU (B), or TMZ (C), and proliferation was determined using the XTT assay. The values are the means ± S.D. of at least three independent experiments. D, TMZ-induced apoptosis. Cells were incubated for 24 h with various concentrations (1, 10, 100, and 1000 μM) of TMZ, and apoptotic cells were identified using Hoechst 33342 staining. S0, CEM-S cells untreated; S1 to S1000, CEM-S cells treated with 1 to 1000 μM TMZ, respectively; R0, CEM-R cells untreated; R1 to R1000, CEM-R cells treated with 1 to 1000 μM TMZ, respectively. The values are the means ± S.D. of at least three independent experiments. E and F, typical images of nuclei of untreated CEM-R cells (E, not apoptotic) or CEM-R cells treated with 1000 μM TMZ (F, apoptotic).

Cell Cycle Analysis Using Flow Cytometry. Flow cytometric analysis was performed to evaluate cell cycle progression. Samples were fixed in 80% ethanol, stained with 20 μg/ml propidium iodide (Beckman Coulter, Fullerton, CA), and analyzed using a Beckman Coulter Epics XL Flow Cytometer (GMI, Inc., Ramsey, MN).

Western Blot Analysis. Protein lysates were subjected to SDS-polyacrylamide gel electrophoresis on a 10% acrylamide gel, and transferred electrophoretically onto Immobilon-P membranes (Millipore, Billerica, MA). The membranes were probed by standard techniques with the primary antibodies, and then with the secondary antibodies. The enhanced chemiluminescence (ECL) detection kit (Cell Signaling Technology, Danvers, MA) and Hyperfilm ECL (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, UK) were used to visualize the presence of proteins. Rabbit polyclonal anti-Bcl-2, rabbit polyclonal anti-Bad, and rabbit polyclonal anti-Bax (all from Cell Signaling Technology), rabbit polyclonal anti-phosphorylated Akt (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-Pol-β (Kamiya Biomedical Company, Seattle, WA), mouse monoclonal anti-APE (AP endonuclease; R&D Systems Inc., Minneapolis, MN), mouse monoclonal anti-TNF Receptor II/TNFRSF1B (R&D Systems, Inc.), mouse monoclonal anti-p53 (Calbiochem, La Jolla, CA), rabbit polyclonal anti-phosphorylated Rb (Cell Signaling Technology), and rabbit polyclonal anti-actin antibody (Sigma) were used as primary antibodies.

Suppression of hMLH1 by Transfecting a shRNA-Containing Vector into CEM-R Cells. To alter MMR activity, shRNA against hMLH1 was transfected into CEM-R cells. Plasmid DNA was prepared from an *Escherichia coli* clone, which contained shRNA against MLH1 (V2HS_76498; Open Biosystems, Huntsville, AL) and anti-puromycin marker, using the Plasmid Mini kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. Retroviral packaging was performed using the Expression Arrest-LynX retroviral expression system for shRNA (Open Biosystems) according to the manufacturer's instructions. CEM-R cells were then infected with the virus, and one subclone transfected with shRNA against hMLH1 was selected and established as CEM-R-shr by the limiting dilution procedure. The control cells (CEM-R-gfp) that had been transfected with empty vector were also prepared.

TABLE 1

Drug sensitivities of CEM-S and CEM-R cells

CEM-S and CEM-R cells were incubated for 72 h with various concentrations of TMZ, BCNU, or ACNU combined with or without MX, BG, or $CdCl_2$. The IC_{50} was then determined using the XTT assay. Relative resistance values were obtained by dividing the IC_{50} value of CEM-R cells by that of CEM-S cells.

Drugs	IC_{50}		Relative Resistance
	CEM-S	CEM-R	
	μM		
TMZ	1800	201	0.1
BCNU	7	55	7.9
ACNU	8	72	9.0
TMZ + MX	120	230	
BCNU + MX	8	49	
TMZ + BG	1450	4	
BCNU + BG	7	16	
TMZ + $CdCl_2$	1550	1300	
BCNU + $CdCl_2$	9	28	

Results

DNA Repair- and Apoptotic and Survival Pathway-Related Proteins Were Involved in BCNU Resistance. Enhanced DNA excision repair activity including NER was associated with the development of BCNU resistance in CEM-R cells (Yamauchi et al., 2003), but the mechanisms of resistance to a given anticancer agent are generally multifactorial. As determined by real-time RT-PCR, the MGMT transcript was detected in CEM-R cells but not in CEM-S cells (Fig. 1A), suggesting a critical role of this enzyme in repairing O^6-alkylguanine produced by BCNU. On the other hand, GSTπ mRNA level was comparable in both cells, suggesting that it did not contribute to detoxification of BCNU (Fig. 1B). The mRNA levels of the MMR-related proteins hMLH1 and hMSH2 were 2-fold greater in CEM-R cells than in CEM-S cells (Fig. 1, C and D), suggesting that MMR helped protect the cells against interstrand cross-links generated by BCNU (Aquilina et al., 1998; Fiumicino et al., 2000;

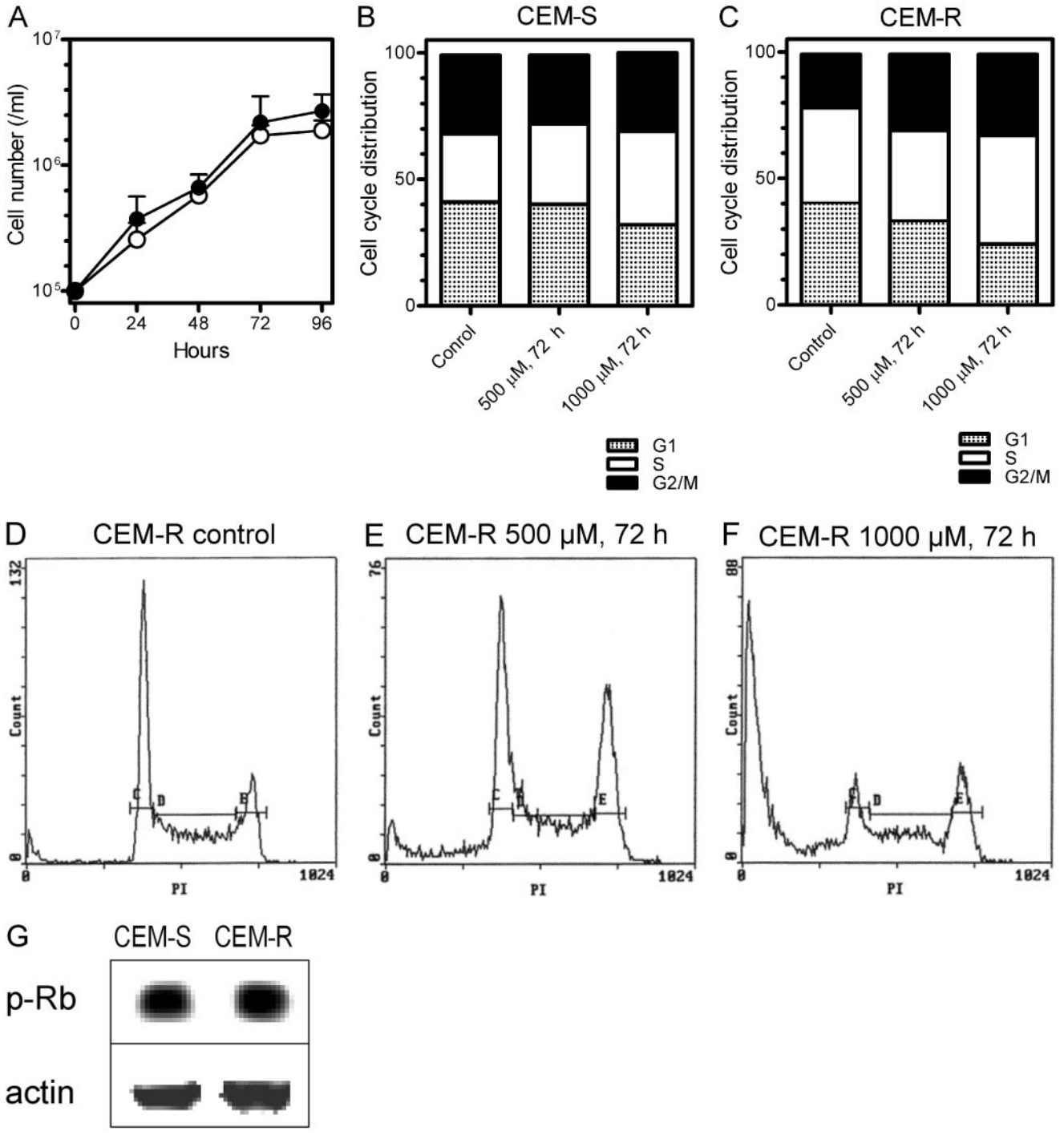

Fig. 3. Cell cycle distribution of CEM-S and CEM-R cells treated with TMZ. A, doubling times were 18.2 h for CEM-S (○) and 17.2 h for CEM-R (●) cells. B–F, cell cycle analysis. CEM-S (B) and CEM-R (C–F) cells were treated with or without TMZ at 500 or 1000 μM for 72 h, followed by flow cytometric analysis. G, Western blot analyses of whole-cell lysates using an antibody against phosphorylated Rb.

Pepponi et al., 2003). Moreover, Western blotting revealed that anti-apoptotic Bcl-2 was enhanced in CEM-R cells, whereas pro-apoptotic Bax and Bad were comparable between CEM-S and CEM-R cells (Fig. 1E). TNF receptor was not detected in either cell by Western blotting (data not shown). In addition, AKT was more phosphorylated in CEM-R cells than CEM-S cells, suggesting enhanced prototypic survival pathway (Fig. 1F). Thus, these results suggested that the mechanisms of BCNU resistance in CEM-R cells further involved DNA repairs including MGMT and MMR and apoptotic and survival pathways.

CEM-R Cells Were More Sensitive to TMZ Than Were CEM-S Cells. The IC_{50} values for TMZ, BCNU, and ACNU were determined. CEM-R cells were more resistant to BCNU and a similar bifunctional agent ACNU than were CEM-S cells (Fig. 2, A and B; Table 1). CEM-S cells were not sensitive to TMZ, probably because of the low level of their MMR activity (Taverna et al., 2000; Matheson and Hall, 2003). It is surprising, however, that CEM-R cells were 1-log more sensitive to TMZ than were CEM-S cells (Fig. 2C and Table 1). CEM-R cells were also more susceptible to TMZ-induced apoptosis despite enhanced Bcl-2 and AKT (Fig. 2, D–F). Thus, the cytotoxic effect of TMZ was greater on BCNU-resistant CEM-R cells than on BCNU-sensitive CEM-S cells.

TMZ Induced G_2/M Arrest in CEM-R Cells. Cell cycle distribution was analyzed for CEM-S and CEM-R cells after treatment. Despite the comparable doubling time for both cell lines (Fig. 3A), G_2/M arrest was induced in CEM-R cells at 72 h after treatment with 500 μM TMZ (Fig. 3, C–F), which might be in accordance with the finding in previous reports (Ma et al., 2002). For CEM-S cells, however, the cell cycle distribution was largely unchanged by the higher concentration (1000 μM) of TMZ (Fig. 3B). Proteins related to cell cycle also were examined. Phosphorylation of Rb protein was comparable between CEM-S and CEM-R cells (Fig. 3G) but p53 was not detected in either cell line by Western

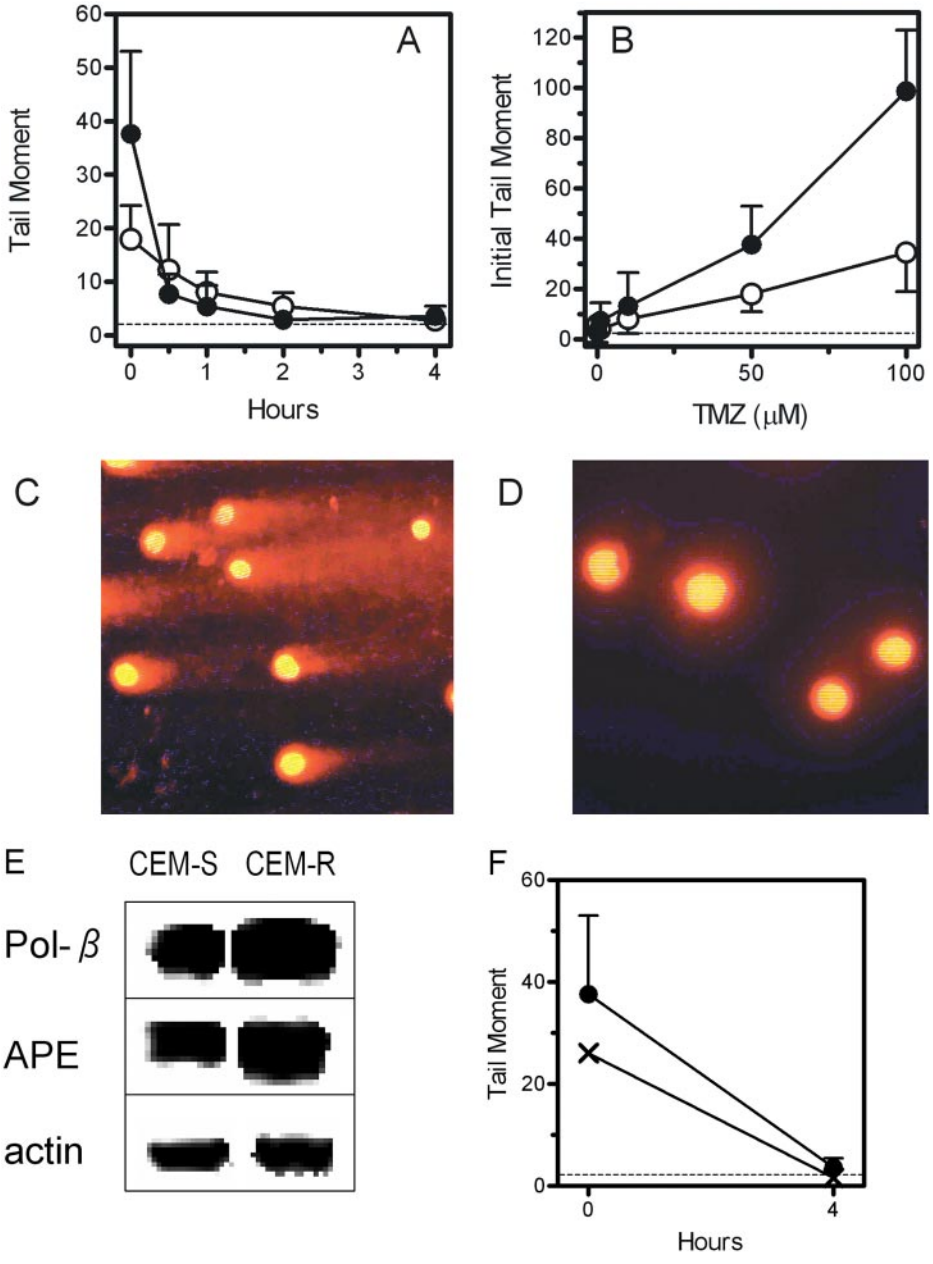

Fig. 4. DNA excision repairs in response to TMZ-induced DNA damage. A, CEM-S (○) and CEM-R (●) cells were pulsed with 50 μM TMZ for 1 h, followed by washing in fresh media and incubation. The samples were taken at indicated time points (0, 0.5, 1, 2, and 4 h) for the Comet assay. B, CEM-S (○) and CEM-R (●) cells were incubated with various concentrations of TMZ for 1 h, immediately followed by Comet assay evaluation. The values are the means ± S.D. of at least three independent experiments. The dotted lines represent control levels. The actual Comet figures at 0 h (C) and 4 h (D) were shown after CEM-R cells had been treated with 50 μM TMZ. E, Western blot analyses of whole-cell lysates using antibodies against Pol-β and APE. F, BER in response to TMZ-induced DNA damage. CEM-R cells were pulsed with 50 μM TMZ for 1 h with or without BER inhibitor MX (6 mM), followed by washing in fresh media and used in the Comet assay (0 h). An aliquot underwent subsequent incubation with MX (6 mM), followed by the Comet assay (4 h). The values are the means ± S.D. of at least three independent experiments. The dotted line represents the control level. ●, CEM-R cells treated with TMZ; ×, CEM-R cells treated with TMZ + MX.

blotting (data not shown). Thus, these results suggested that sensitization to TMZ was associated with the effective cell cycle arrest induced by TMZ in CEM-R cells.

DNA Excision Repairs Initiated by TMZ Were More Efficient in CEM-R Cells. TMZ produces N^7-alkylguanine (70%), N^3-alkyladenine (9%), and O^6-alkylguanine (5%) in cellular DNA (Newlands et al., 1997). The former two adducts account for the majority of the alkylation products, which are primarily removed by DNA excision repairs including NER and BER (Plosky et al., 2002; Drabløs et al., 2004). To determine the cellular response to TMZ-induced N^7-alkylguanine and N^3-alkyladenine, DNA excision repair kinetics were compared between CEM-S and CEM-R cells. The process of DNA excision repair consists of incision and excision of the damaged nucleotides by endonucleases (generation of DNA strand breaks), gap filling by resynthesis of DNA, and rejoining by ligation (decrease and disappearance of the breaks), the kinetics of which can be quantitated as the amount of DNA strand breaks by the Comet assay (Yamauchi et al., 2001, 2002, 2003). When cells were pulsed with 50 μM TMZ, followed by washing in fresh media and subsequent incubation, the tail moment (the amount of DNA strand breaks) was greatest at the end of the pulse period (0 h) (Figs. 4, A and C), which then decreased rapidly and returned to the control level within 4 h (Fig. 4, A and D). This suggested that the incision and excision step occurred during the pulse period, with the resulting gap rapidly filled by DNA resynthesis and DNA repair completed within 4 h after treatment. The process seemed to be most linear between 0 and 1 h, with the slope leveling thereafter (Fig. 4A), suggesting that the value of the 0 h/1 h tail moment ratio represented the rate of the repair. The rate of the repair in CEM-R cells (6.0) was almost double that in CEM-S cells (2.6), suggesting acceleration of the repair rate in CEM-R cells. When the cells were treated with different concentrations of TMZ, the initial tail moment (the tail moment at the end of the incubation period) increased in a concentration-dependent manner (Fig. 4B), reflecting the cellular incision/excision repair activity in response to TMZ-induced N^7-alkylguanine and N^3-alkyladenine. These initial tail moments were greater in CEM-R cells than in CEM-S cells (Fig. 4B), suggesting an enhanced incision/excision activity in CEM-R cells. Taken together, the present data indicated that CEM-R cells had greater DNA excision repair activity than CEM-S cells, the results of which were compatible with previous findings using BCNU (Yamauchi et al., 2003). TMZ-induced N^7-alkylguanine and N^3-alkyladenine therefore seemed to be repaired more efficiently in CEM-R cells than in CEM-S cells, suggesting that these adducts were not associated with the increased sensitivity of CEM-R cells to TMZ.

BER was evaluated more specifically. The protein expres-

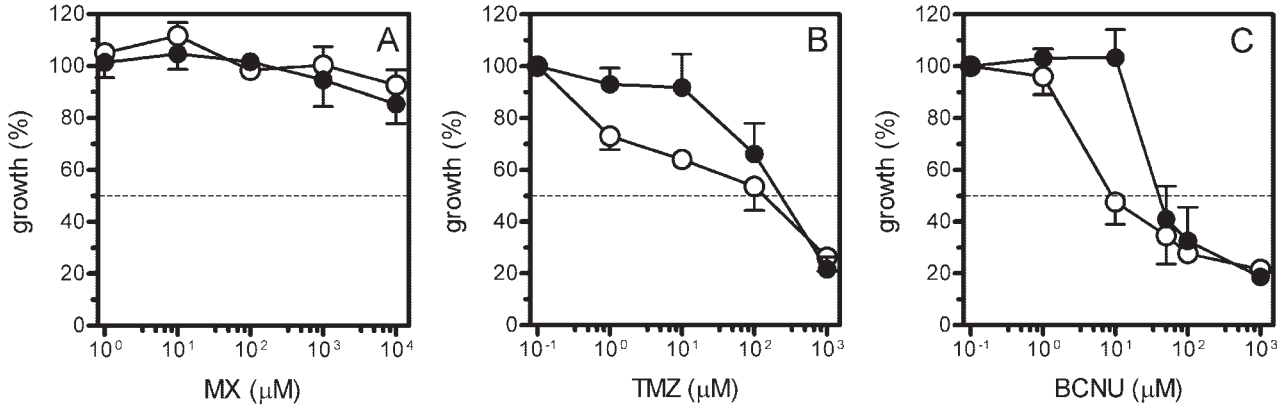

Fig. 5. Effect of MX, an inhibitor of BER. CEM-S (○) and CEM-R (●) cells were incubated for 72 h with a minimally toxic concentration (6 mM) of MX and with various concentrations of TMZ or BCNU. A, MX alone. B, MX plus TMZ. C, MX plus BCNU. The cell growth was determined using the XTT assay. The values are the means ± S.D. of at least three independent experiments.

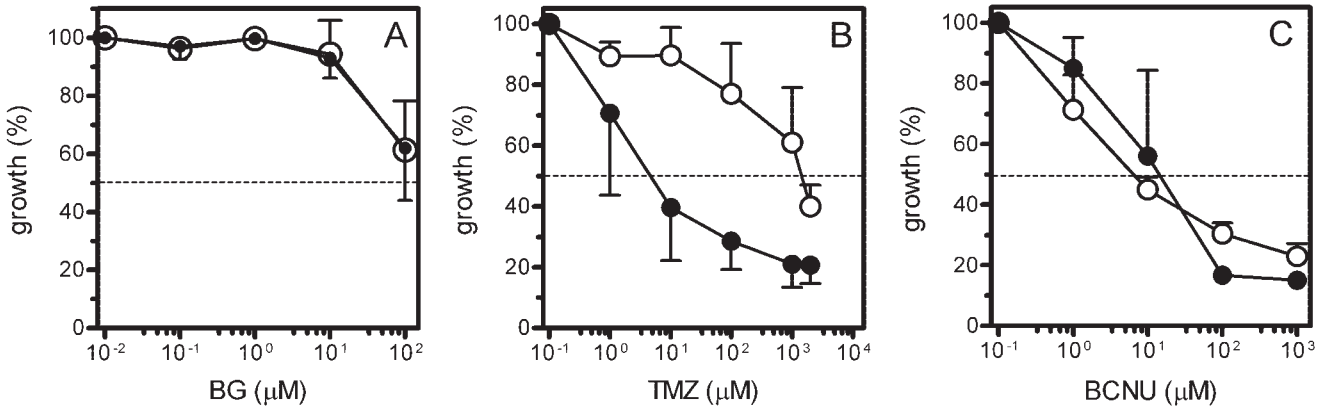

Fig. 6. Effect of BG, an inhibitor of MGMT. CEM-S (○) and CEM-R (●) cells were preincubated for 2 h with a minimally toxic concentration (10 μM) of BG, followed by coincubation for 72 h with various concentrations of TMZ or BCNU. A, BG alone. B, BG plus TMZ. C, BG plus BCNU. The cell growth was determined using the XTT assay. The values are the means ± S.D. of at least three independent experiments.

sion of two BER-related proteins Pol-β and APE were augmented in CEM-R cells compared with CEM-S cells, suggesting enhanced BER activity after selection of the cells acquiring BCNU resistance (Fig. 4E). To further specify the activity of BER, we did another Comet assay using BER inhibitor MX (Fig. 4F). When CEM-R cells were treated with TMZ and with or without MX, the initial tail moment was reduced by the addition of MX (Fig. 4F), suggesting the participation of BER in repairing TMZ-induced *N*-alkylation adducts. However, MX did not alter the tail moment value at 4 h (Fig. 4F), which returned to the control level, indicating the completion of excision repairs. This suggested that NER compensated BER activity, thereby repairing *N*-alkylation adducts, and therefore *N*-alkylated products were not cytotoxic despite the alteration of BER activity. Thus, it was suggested that BER was not crucial to the sensitivity of CEM-R cells to TMZ despite enhanced BER-related protein levels.

Inhibition of BER Did Not Sensitize CEM-R Cells to TMZ. To further evaluate the cytotoxicity of N^7-alkylguanine and N^3-alkyladenine, MX was again used in combination with TMZ. When CEM-R cells were incubated with TMZ plus MX at a concentration minimally toxic to the cells (Fig. 5A) but effective for inhibiting BER (Liu et al., 1999; Rinne et al., 2004), the growth inhibitory effect of TMZ was not augmented (Fig. 5, B and Table 1), suggesting that *N*-alkylated purines were not sufficient for inducing cytotoxicity in CEM-R cells. To the contrary, the addition of MX sensitized CEM-S cells to TMZ markedly (Fig. 5B, Table 1), suggesting that TMZ-induced *N*-alkylation products were potentially cytotoxic to CEM-S cells. This result might be in accordance with a previous report demonstrating that *N*-alkylation products became more cytotoxic than O^6-alkylguanine in TMZ-treated cells that had a low level of MMR (Liu et al., 1999). The cytotoxic effect of BCNU was not altered by adding MX in both cell types (Fig. 5C, Table 1), suggesting that BER was not involved in repairing interstrand cross-links, the primary lesions responsible for BCNU cytotoxicity. Thus, these results suggested that N^7-alkylguanine and N^3-alkyladenine did not contribute to TMZ cytotoxicity in CEM-R cells.

Inhibition of MGMT Markedly Sensitized CEM-R Cells to TMZ. Because the O^6-alkylguanine-mediated mismatch triggers the MMR response and because the formation of interstrand cross-links proceeds through the intermediate

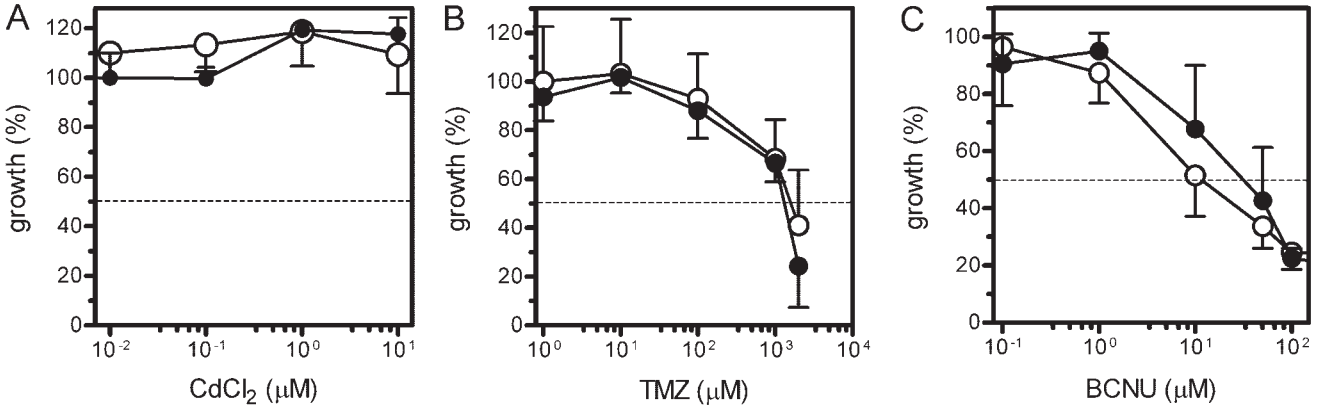

Fig. 7. Effect of $CdCl_2$, an inhibitor of MMR. CEM-S (○) and CEM-R (●) cells were preincubated for 2 h with a minimally toxic concentration (10 μM) of $CdCl_2$, followed by coincubation for 72 h with various concentrations of TMZ or BCNU. A, $CdCl_2$ alone. B, $CdCl_2$ plus TMZ. C, $CdCl_2$ plus BCNU. The cell growth was determined using the XTT assay. The values are the means ± S.D. of at least three independent experiments.

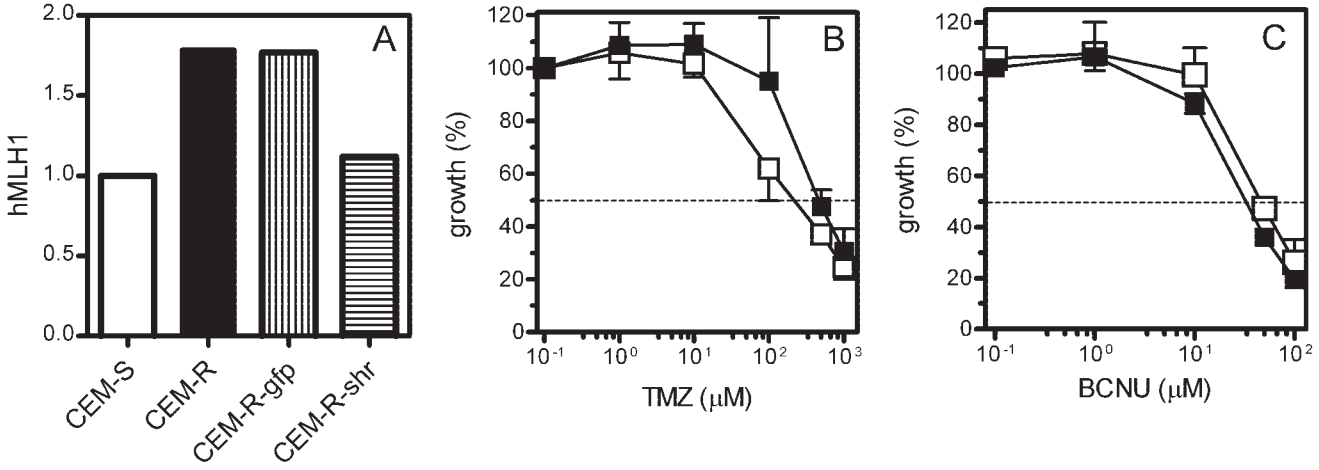

Fig. 8. A, transcript levels of hMLH1 in a CEM-R variant (CEM-R-shr) transfected with shRNA against hMLH1, the control (CEM-R-gfp) transfected with empty vector, CEM-S, and CEM-R cells. B and C, growth inhibition curves of CEM-R-shr (■) cells and CEM-R-gfp (□) cells. Cells were incubated for 72 h with TMZ (B) or BCNU (C), and proliferation was determined using the XTT assay. The values are the means ± S.D. of at least three independent experiments.

production of O^6-alkylguanine, MGMT is closely associated with the mechanisms of resistance to both TMZ and BCNU. The presence of MGMT transcripts in CEM-R cells (Fig. 1A) suggested an efficient repair of O^6-alkylguanine by this suicide protein. When CEM-R cells were incubated with TMZ plus BG, an inhibitor of MGMT, at a concentration minimally toxic to the cells (Fig. 6A) but effective for inhibiting MGMT (Bobola et al., 2005), cell growth was inhibited quite potently (Fig. 6B, Table 1). An almost 50-fold reduction of the IC_{50} value compared with that by TMZ alone was observed (Table 1), strongly suggesting that O^6-alkylguanine was crucial for the cytotoxic effects of TMZ on CEM-R cells. BG also enhanced the growth inhibitory effect of BCNU on CEM-R cells (Fig, 6C, Table 1), suggesting the participation of functional MGMT in the mechanism of resistance to BCNU in CEM-R cells. Conversely, BG was not effective for sensitizing CEM-S cells to TMZ and BCNU (Figs. 6, B and C; Table 1), which might be attributable to the lack of MGMT in CEM-S cells. Thus, these results suggested that O^6-alkylguanine was the primary lesion responsible for TMZ-induced cytotoxicity in CEM-R cells.

Inhibition of MMR Disrupted Sensitivity of CEM-R Cells to TMZ. TMZ cytotoxicity depends upon MMR function triggered by O^6-alkylguanine, which may explain why CEM-S cells that naturally have a low level of MMR activity are not so sensitive to TMZ (Fig. 2C, Table 1). Because CEM-R cells have increased mRNA levels of hMLH1 and hMSH2, TMZ-induced O^6-alkylguanine that surpassed even the enhanced MGMT capacity in the cells may induce the MMR response, thereby exerting the cytotoxicity. When CEM-R cells were treated with TMZ plus $CdCl_2$, an inhibitor of MMR, at a concentration minimally toxic to the cells (Fig. 7A) but effective for inhibiting MMR (Jin et al., 2003; Lützen et al., 2004), the sensitivity to TMZ was disrupted with the IC_{50} value close to that of TMZ alone for CEM-S cells (Fig. 7B, Table 1). This suggested that MMR played a critical role in the sensitivity of CEM-R cells to TMZ. Conversely, the addition of $CdCl_2$ sensitized CEM-R cells to BCNU partially (Fig. 7C, Table 1), suggesting that MMR was involved in the development of BCNU-resistance. The sensitivity of CEM-S cells to TMZ or BCNU was not altered by cotreatment with $CdCl_2$ (Fig. 7, B and C, Table 1). Thus, these results suggested that MMR was closely associated with CEM-R cells' enhanced sensitivity to TMZ.

Suppression of hMLH1 Partially Reversed Sensitivity of CEM-R Cells to TMZ. To further investigate the role of MMR in the sensitivity of CEM-R cells to both TMZ and BCNU, shRNA against hMLH1 was transfected into CEM-R cells. CEM-R-shr cells, the CEM-R variant clone transfected with anti-hMLH1 shRNA, demonstrated a reduced mRNA level of hMLH1 (Fig. 8A), which was almost equivalent to the level in CEM-S cells. The sensitivity of CEM-R-shr cells to TMZ was partially reversed with a 2.5-fold increase in the IC_{50} value (Fig. 8B, Table 2), strongly suggesting that the increased sensitivity of CEM-R cells to TMZ was due to enhancement of MMR. Moreover, BCNU resistance was partially reversed in CEM-R-shr cells as well (Fig. 8C, Table 2), suggesting that MMR was in part responsible for the development of cellular resistance to BCNU. The sensitivity to TMZ or BCNU was not altered in CEM-R-gfp, the control cells that had been transfected with empty vector (Fig. 8, A–C; Table 2). Thus, these results suggested that the improved sensitivity of CEM-R cells to TMZ was attributable to MMR that might be enhanced by up-regulation of hMLH1 and hMSH2 transcript levels during the development of BCNU resistance.

Transcript Levels of MMR-Related Proteins Were Associated with TMZ Sensitivity in Different Cell Lines. To confirm these unique events demonstrated using CEM-S and CEM-R cells, different cell lines were examined for the relationship between transcript levels of hMLH1 and hMSH2 and drug sensitivity. When transcript levels of hMLH1 and hMSH2 were low, the sensitivity to TMZ was relatively low compared with BCNU sensitivity, as was the case with K562, CCRF-697, and U266 cells (Table 3). Conversely, RPMI8226 cells, which had the higher transcript levels, possessed greater sensitivity to TMZ relative to BCNU than did the above three cell lines (Table 3). These results thus suggested the correlation between MMR-related factor levels and cellular sensitivity to TMZ relative to BCNU.

TABLE 2

Drug sensitivities of shRNA-transfected CEM-R cells

Both CEM-R-shr cells that were transfected with shRNA against hMLH1 and the control CEM-R-gfp cells that were transfected with empty vector were incubated with various concentrations of TMZ or BCNU for 72 h. The IC_{50} was then determined using XTT assay. Relative resistance values were obtained by dividing the IC_{50} value of CEM-R-shr cells by that of CEM-R-gfp cells.

Drugs	IC_{50}		Relative Resistance
	CEM-R-gfp	CEM-R-shr	
	μM		
TMZ	204	500	2.5
BCNU	50	30	0.6

Discussion

We demonstrated that BCNU-resistant leukemic CEM-R cells were more sensitive to TMZ than were BCNU-sensitive CEM-S cells (Fig. 2). Although TMZ is mainly used for treating brain tumor, it may be possible to apply this agent to leukemic cell lines because recent trials evaluated its clinical efficacy against leukemia (Brandwein et al., 2007; Horton et

TABLE 3

Transcript levels of DNA repair proteins and drug sensitivity in different cell lines

Relative resistance is the ratio of the IC_{50} values of TMZ to BCNU in K562 and U266 cells or the ratio of TMZ + BG to BCNU + BG in RPMI8226 and CCRF-697 cells.

Cell Line	mRNA Levels			IC_{50}				Relative Resistance
	MGMT	hMLH1	hMSH2	BCNU	BCNU + BG	TMZ	TMZ + BG	
				μM				
RPMI8226	0.06	0.66	0.84	30.0	5.0	160.0	2.4	0.5
K562	0.00	0.44	0.28	28.6		36.0		1.2
CCRF-697	0.16	0.23	0.26	5.2	0.3	42.4	0.4	1.3
U266	0.00	0.29	0.44	37		1200		32

al., 2007). The mechanisms of BCNU-resistance in CEM-R cells involved DNA repairs including DNA excision repairs, MGMT and MMR, and apoptotic and survival pathways (Figs. 1–4). Among these, we focused on DNA repair to determine the mechanism of action of TMZ in the context of BCNU resistance, because apoptotic and survival pathways may affect the sensitivity to both agents equally.

TMZ creates a variety of DNA adducts, including N^7-alkylguanine, N^3-alkyladenine, and O^6-alkylguanine (Newlands et al., 1997), all of which may contribute to TMZ cytotoxicity. *N*-Alkylated products account for almost 80% of all adducts and are potentially cytotoxic. They are substrates for DNA excision repair pathways including NER and BER (Plosky et al., 2002; Drabløs et al., 2004), and the Comet assay clearly demonstrated that CEM-R cells repaired these adducts more efficiently than did CEM-S cells (Fig. 4). This strongly suggested that the *N*-alkylated products were not associated with the increased sensitivity of CEM-R cells to TMZ. This was supported by the experiment using MX, an inhibitor of BER, which did not augment the sensitivity of CEM-R cells to TMZ (Figs. 4 and 5, Table 1). Even though it is only a minor adduct, O^6-alkylguanine is believed to be the most important lesion for TMZ-induced cytotoxicity. In CEM-R cells, in which MGMT expression was induced (Fig. 1A), BG, an inhibitor of MGMT, reversed BCNU resistance partially (Fig. 6, Table 1), indicating the presence of functional MGMT that contributed to BCNU resistance. More importantly, BG markedly augmented the sensitivity of CEM-R cells to TMZ (Fig. 6, Table 1), thus strongly suggesting that O^6-alkylguanine was the major cytotoxic lesion generated by TMZ in this cell line.

Regarding O^6-alkylguanine as the most critical DNA damage, both the efficacy of prereplicative repair of this adduct by MGMT and postreplicative MMR activity determine the level of TMZ cytotoxicity in CEM-R cells (Barvaux et al., 2004). CEM-S cells that naturally had a low level of MMR activity (Taverna et al., 2000; Matheson and Hall, 2003) and lacked MGMT mRNA (Fig. 1A) were not sensitive to TMZ (Fig. 2, C and D, and Table 1). This suggested that even unrepaired O^6-alkylguanine was not cytotoxic in the absence of MMR (Fink et al., 1998). Compared with CEM-S cells, CEM-R cells acquired up-regulated transcript levels of hMLH1 and hMSH2 (Fig. 1, C and D) and were much more sensitive to TMZ despite the presence of MGMT (Fig. 2, C and D, Table 1). Western blot and ELISA techniques did not detect the proteins of hMLH1 and hMSH2 in both CEM-S and CEM-R cells (data not shown), because of their low protein levels (Taverna et al., 2000; Matheson and Hall, 2003). Nevertheless, $CdCl_2$, an inhibitor of MMR, disrupted TMZ sensitivity in CEM-R cells (Fig. 7, Table 1). Furthermore, the reduction of the hMLH1 transcript level reversed the cellular sensitivity to TMZ partially in CEM-R-shr cells, a CEM-R-derived clone transfected with shRNA against hMLH1 (Fig. 8, Table 2). These results thus suggested that MMR, which seemed to be enhanced during the development of BCNU resistance, sensitized CEM-R cells to TMZ.

MMR involves multiple proteins, which recognize the mismatch, excise the DNA containing the error, and resynthesize the correct DNA sequence (Kolodner and Marsischky 1999). In eukaryotes, the initial recognition of mispairs is carried out by the hMSH2-hMSH6 heterodimer, which recognizes base-base mismatches, or the hMSH2-hMSH3 heterodimer, which recognizes frame shifts and larger insertion/deletion mismatches. hMLH1, PMS2, and hMLH3 form the second heterodimers, which participate in downstream events. hMSH2 and hMLH1 are therefore regarded as key factors in MMR. Cadmium inhibits the hMSH2-hMSH6 protein, thereby abrogating the MMR pathway (Jin et al., 2003; Clark and Kunkel, 2004; Lützen et al., 2004; Banerjee and Flores-Rozas, 2005). Because TMZ-induced mismatches are recognized by the hMSH2-hMSH6 MutSα heterodimer (Yoshioka et al., 2006), cadmium may inhibit the recognition process and interfere with MMR function, leading to the inhibition of TMZ cytotoxicity. Our findings (Fig. 7, Table 1) were consistent with these reports. The reduction in hMLH1 expression using shRNA in CEM-R-shr cells was less effective than the use of $CdCl_2$ in CEM-R cells for disrupting the sensitivity to TMZ (Fig. 8, Table 2). Several reports suggest that hMLH1 and hMSH2 may affect cells' sensitivity to TMZ differently (Marra et al., 2001; Sansom et al., 2001), which might explain the difference in the effects between $CdCl_2$ that may inhibit the process where hMSH2 is involved (Fig. 7, Table 1) and shRNA against hMLH1 (Fig. 8, Table 2).

It has been suggested that MMR participates in the removal of the interstrand cross-links. Hypersensitivity to bifunctional alkylator CCNU and a similar cross-linking agent mitomycin C were demonstrated in cells that lacked MMR (Aquilina et al., 1998; Fiumicino et al., 2000). An inverse correlation was shown between BCNU sensitivity and MMR activity in 11 melanoma cell lines, indicating that melanoma cells were protected against BCNU in part by the MMR system (Pepponi et al., 2003). The participation of MMR was also demonstrated in error-free processing of psoralen-mediated cross-links (Reddy and Vasquez, 2005; Wu et al., 2005). Here, CEM-R cells, which had up-regulated hMLH1 and hMSH2 mRNA levels, exhibited partial restoration of the cellular sensitivity to BCNU by cotreatment with $CdCl_2$ or by the transfection of shRNA against hMLH1. The results thus suggested a possible contribution of functional MMR to BCNU resistance in CEM-R cells.

In conclusion, cancer cells that had become resistant to BCNU were sensitized to TMZ as a result of enhancement of MMR during the development of BCNU resistance. Based upon such a mechanistic interaction between the drug and DNA repair, a chemotherapy regimen including TMZ may be advisable for treating patients with recurrent brain tumors that have become resistant to prior chemotherapy using bifunctional nitrosourea such as BCNU. Vice versa, BCNU might be effective against the tumors that recur after TMZ treatment.

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