Cancer Therapy: Preclinical

Temozolomide-Mediated DNA Methylation in Human Myeloid Precursor Cells: Differential Involvement of Intrinsic and Extrinsic Apoptotic Pathways

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Abstract

**Purpose:** An understanding of how hematopoietic cells respond to therapy that causes myelosuppression will help develop approaches to prevent this potentially life-threatening toxicity. The goal of this study was to determine how human myeloid precursor cells respond to temozolomide (TMZ)-induced DNA damage.

**Experimental Design:** We developed an *ex vivo* primary human myeloid precursor cells model system to investigate the involvement of cell-death pathways using a known myelosuppressive regimen of O6-benzylguanine (6BG) and TMZ.

**Results:** Exposure to 6BG/TMZ led to increases in p53, p21, γ-H2AX, and mitochondrial DNA damage. Increases in mitochondrial membrane depolarization correlated with increased caspase-9 and -3 activities following 6BG/TMZ treatment. These events correlated with decreases in activated AKT, downregulation of the DNA repair protein O6-methylguanine–DNA methyltransferase (MGMT), and increased cell death. During myeloid precursor cell expansion, FAS/CD95/APO1(FAS) expression increased over time and was present on approximately 100% of the cells following exposure to 6BG/TMZ. Although c-flipshort, an endogenous inhibitor of FAS-mediated signaling, was decreased in 6BG/TMZ–treated versus control, 6BG-, or TMZ-alone–treated cells, there were no changes in caspase-8 activity. In addition, there were no changes in the extent of cell death in myeloid precursor cells exposed to 6BG/TMZ in the presence of neutralizing or agonistic anti-FAS antibodies, indicating that FAS-mediated signaling was not operative.

**Conclusions:** In human myeloid precursor cells, 6BG/TMZ–initiated apoptosis occurred by intrinsic, mitochondrial-mediated and not extrinsic, FAS-mediated apoptosis. Human myeloid precursor cells represent a clinically relevant model system for gaining insight into how hematopoietic cells respond to chemotherapeutics and offer an approach for selecting effective chemotherapeutic regimens with limited hematopoietic toxicity. *Clin Cancer Res; 19(10); 2699–709. ©2013 AACR.*

Introduction

A major dose-limiting toxicity in anticancer chemotherapy is the induction of persistent DNA damage that leads to programmed cell death of hematopoietic cells in the blood, spleen, and bone marrow (1). In addition, the survival of rare hematopoietic-derived clonal populations with transforming DNA mutations due to chemotherapy exposure can lead to emergence of leukemic cells (2). A primary contributing factor responsible for these deleterious outcomes is that hematopoietic cells typically express low levels of DNA repair proteins, and therefore are highly susceptible to DNA damage caused by therapeutics targeting cancer cells (3, 4). Understanding molecular processes that regulate how primary human hematopoietic cells respond to DNA damage could provide key information toward development of cancer treatments that specifically target cancer cells with minimal effects to normal hematopoietic cells.
Translational Relevance

In response to chemotherapy treatment, patients with cancer can experience adverse side effects due to myelosuppression as well as increased propensity to develop secondary hematologic malignancies. However, model systems designed to study molecular events initiated by chemotherapy-mediated toxicity in immature hematopoietic cells are lacking. To address this important clinical issue, we examined the DNA-damage response of human myeloid precursor cells exposed to a temozolomide (TMZ)-based regimen known to cause myelosuppression in patients. Activation of the FAS/CD95/APO1- versus the mitochondrial-mediated cell-death pathway was investigated. When TMZ-induced DNA damage persisted in the nuclear and mitochondrial genomes, apoptosis of myeloid precursor cells was induced by the mitochondrial- but not the FAS-mediated cell-death pathway. Primary human myeloid precursor cells represent a clinically relevant approach to investigate how nontransformed primary hematopoietic cells respond to chemotherapy-induced DNA damage. Understanding the molecular basis of myelosuppression will aid in the development and design of more effective chemotherapies for cancer treatment.

Myeloid cells represent a diverse population of hematopoietic cells consisting of granulocyte and monocyte/macrophage lineages derived from pluripotent hematopoietic stem cells (1, 2). Upon maturation, myeloid cells play critical roles in regulating immune responses, bone remodeling, and inflammation. Therefore, if left unrepaired, chemotherapy-mediated DNA damage can be highly detrimental to myeloid cell function. In this study, we examine the response of human myeloid precursor cells to temozolomide (TMZ), as it is routinely used as a first-line chemotherapeutic agent for the treatment of glioblastoma multiforme (5). In particular, the molecular effects of TMZ-mediated myelosuppression in the presence of the O6-methylguanine–DNA methyltransferase (MGMT) inhibitor, O6-benzylguanine (6BG), were studied as (i) myelosuppression is observed in the clinic with this regimen; and (ii) dependence of DNA repair and cell survival on MGMT expression could be assessed pharmacologically (6, 7). TMZ is a pro-drug that hydrolyzes to its active metabolite (3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC) at physiologic pH (8). The main mechanism of TMZ-mediated cytotoxicity is the generation of a variety of DNA adducts including N2-methylguanine, N3-methyladenine, and O6-methylguanine (O6MeG). However, how the presence of methylated adducts leads to cell death is complex and not completely understood (9). Although the base excision repair system is responsible for repairing N2-methylguanine and N3-methyladenine adducts, the direct repair protein, MGMT, repairs O6MeG adducts. If left unrepaired, the O6MeG adduct can be highly cytotoxic and is the most critical DNA lesion contributing to cell death when cells are exposed to alkylating reagents such as TMZ. This adduct can mispair with a thymine instead of the cytosine residue during DNA replication, which leads to the formation of O6MeG:thymine mismatches. Although the mismatches are recognized by the mismatch repair (MMR) system (10), a futile cycle of repair ensues in which thymine is excised only to have another thymine reinserted opposite of the O6MeG adduct. This continues as long as O6MeG adducts are present and eventually leads to increased double-strand DNA breaks and ultimately cell death. O6MeG adducts can be directly repaired by MGMT by transfer of the methyl group from the oxygen in guanine to cysteine residue-145 in the active site of MGMT (9). When cells with nonrepaired O6MeG adducts enter DNA replication in the absence of adequate DNA repair, replication stalls at the O6MeG adducts resulting in an increase in double-strand DNA breaks and ultimately apoptosis.

The overall hypothesis of the present study is that persistence of TMZ-mediated DNA damage in human myeloid precursor cells results in the activation of a predominant cell-death pathway. To address this hypothesis, we first developed a primary hematopoietic culture system of human origin that could be used to investigate regulation of signaling pathways following exposure to DNA-damaging agents. Human myeloid precursor cells were chosen as these cells represent a population of bone-marrow precursor cells responsible for development of all myeloid lineages. Large numbers of myeloid precursor cells were efficiently generated by incubating human CD34+ cells with granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF). Under these conditions, CD34+ cells underwent robust proliferation starting at day 3 and partial differentiation down the myeloid lineage differentiation pathway by days 7 to 10. We chose to focus on DNA-damage responses elicited via a myelosuppressive TMZ-based regimen previously used in the clinic (6). In this system, we were able to examine a variety of DNA-damage and cell-death response pathways. As a result of 6BG/TMZ treatment, the p53 pathway was activated and DNA damage in both the mitochondrial and nuclear genomes increased. Increased genome damage correlated with MGMT downregulation and increased apoptosis of 6BG/TMZ–treated myeloid precursor cells. In contrast to myeloid precursor cells treated with vehicle control, 6BG, or TMZ, FAS expression increased and expression of the endogenous caspase-8 inhibitor, c-FLIP shorter, decreased in 6BG/TMZ–treated myeloid precursor cells. However, caspase-8 activity remained unchanged and anti-FAS antibodies (agonistic and neutralizing) had no effect on 6BG/TMZ–mediated myeloid precursor cell death indicating that death-inducing signals are not delivered through FAS. In contrast, the intrinsic, mitochondrial-mediated cell-death pathway was activated in myeloid precursor cells as mitochondrial membrane depolarization and caspase-9 activity increased following 6BG/TMZ exposure.
Materials and Methods

Isolation of umbilical cord blood CD34+ cells and expansion of primary human myeloid precursor cells

All protocols were approved by Indiana University School of Medicine’s Institutional Review Board (IRB) and St. Vincent Hospital’s IRB (Indianapolis, IN). Samples of umbilical cord blood (UCB) were collected from normal, full-term infants delivered by cesarean section and the CD34+ cells isolated using the CD34 MicroBead Kit and VarioMACS Separator (Miltenyi Biotech Inc.) according to the manufacturer’s instructions. Isolated CD34+ cells were plated at 0.5 × 10^6 cells/mL and expanded in the presence of 100 ng/mL G-CSF and SCF (PeproTech) for 8 to 12 days in BioWhittaker X-vivo 10 serum-free medium (Lonza) containing 1% human serum albumin.

Formulation of O6-benzylguanine and TMZ

O6-benzylguanine (Sigma) was dissolved in 40% polyethylene glycol-400 (PEG; v/v) and 60% PBS (v/v) and sonicated; final stock concentration was 8.3 mmol/L. The 40% PEG formulation served as the vehicle control (control) in all experiments. TMZ (LKT Laboratories, Inc.) was dissolved in PBS and served as the vehicle control (control) in all experiments. The Jurkat T-lymphoblastoid cell line was purchased from American Type Culture Collection and used at low passage.

Colonies-forming unit assays

Colonies-forming unit (CFU) assays were conducted using isolated human CD34+ cells and expanded CB cells (Method-ocut Gf, H4434; Stem Cell Technologies, Inc.) as previously described (11). The cells were seeded in triplicate in 35-mm dishes at concentrations of 2 × 10^3. After 10 to 14 days of incubation at 37°C in 5% CO_2, CFUs–granulocyte-macrophage (CFU-GM), burst-forming units-erythroid (BFU-E), and CFU–granulocyte/erythrocyte/monocyte/megakaryocyte (CFU-GEMM) were enumerated using the Axiovert 25 inverted-light microscope.

Immunophenotyping by flow cytometry

Phenotype of expanded primary human myeloid cells was conducted in the flow cytometry laboratory. Aliquots of 1 to 2 × 10^5 cells per tube were stained with various antibodies (2 μL per sample) for 25 minutes at 4°C in complete medium and washed one time in PBS containing 1% FBS. Samples were analyzed using 4-color flow cytometry. Total cell count was determined and the viability of the sample was analyzed with 7-aminoactinomycin D staining. The antibodies were directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Cy5 (PC5), or PE-Texas Red (ECD). The following antibodies were analyzed: CD45, CD34, HLA-DR, CD117, CD38, CD71, CD33, CD13, CD15, myeloperoxidase, CD64, CD14, CD3, and CD19. All antibodies were purchased from BD Biosciences. Data acquisition was conducted using COULTER FC-500 flow cytometers and CXP software (Beckman Coulter). The cellular debris was excluded before the analysis. The cells of interest were gated on right angle light scatter versus CD45 display.

Determination of cell death

After treatment, apoptotic cells were detected using APC Annexin V/Dead Cell Apoptosis Kit with APC Annexin V and SYTOX Green (Invitrogen). Myeloid precursor cells were resuspended at a concentration of 1 × 10^6 cells/mL in 1X Annexin-binding buffer; 5 μl allopurinol (APC)-Annexin V and 1 μl of the 1 μmol/L SYTOX Green stain working solution were added to each 100 μl cell suspension. The cells were incubated at 37°C in an atmosphere of 5% CO_2 for 15 minutes and then analyzed by flow cytometry. For antibody-blocking studies of FAS-mediated signaling, the agonistic anti-FAS clone CH-11 (EMD Millipore) and the blocking anti-FAS antibody ZB4 (Kamiya Biomedical) were used. The Jurkat T-lymphoblastoid cell line was purchased from American Type Culture Collection and used at low passage.

Measurement of the mitochondrial membrane potential

Mitochondrial membrane potential was determined by the MitoProbe DiIC(5) Assay Kit for Flow Cytometry (Molecular Probes products; Invitrogen) according to manufacturer’s instructions. Myeloid precursor cells were collected after treatment and incubated with 50 mmol/L DiIC(5) (5) at 37°C, 5% CO_2, for 30 minutes. Cells were washed twice with PBS and analyzed with flow cytometry with 633 nm excitation using emission filters appropriate for Alexa Fluor 633 dye to detect the cyanine dye 1′,3′,3′,3′-hexamethyldiocarbocyanine iodide (DiIC(5); ref. 12). The membrane depolarization agent, carbonyl cyanide m-chlorophenylhydrazone (CCCP), served as a positive control for mitochondrial depolarization.

Cell-cycle analysis

Following treatment, cell-cycle analysis was conducted with FITC BrdU Flow Kits (BD Pharmingen) according to manufacturer’s instructions. Briefly, 10 μL of bromodeoxyuridine (BrdUrd) solution [1 mmol/L BrdUrd in Dulbecco’s PBS (DBPS)] was added to each mL of cell culture media; cell culture density was always maintained at less than 2 × 10^6 cells/mL. The treated cells were incubated for 45 minutes and then fixed and permeabilized with BD Cytofix/Cytoperm Buffer. The cells were then incubated with Dnase followed by 20-minute incubation with fluorescent anti-BrdUrd antibodies to detect incorporation of BrdUrd. Samples were washed and resuspended in buffer. Following the addition of 20 μL 7-aminoactinomycin D solution, the cells were analyzed by flow cytometry.

Western blot analyses

After treatment, the cells were harvested and protein lysates were prepared using 1× Cell Lysis Buffer from Cell Signaling Technology. Because of the large number of proteases in myeloid cells, the following protease inhibitors were included in the lysis buffer: diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, chymostatin, 4′-(2-aminoethyl) benzene sulfonate fluoride hydrochloride, sodium orthovanadate, sodium fluoride (all purchased from

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with identical rectangular regions of interest and subtracting probe correlates with increased DNA damage. The number decrease in the hybridization of the mitochondrial-specific ATPase 6 sequence. After hybridization, membranes were exposed to an imaging screen to determine band intensity. A determination of mitochondrial DNA damage was measured by comparing the optical density (OD) reading from the treated sample with the level of the untreated control. Fold increase in caspase-8 or -9 activity was determined using the Supersignal West Femto Maximum Sensitivity Substrate (Pierce) and exposure to X-ray film (Midwest Scientific).

Caspase-8 and caspase-9 colorimetric activity assay
To measure caspase activity following treatment, the Caspase-8 and Caspase-9 Colorimetric Activity Assay Kits (Chemicon/Millipore) were used according to the manufacturer’s instructions. After treatment, cells were collected and resuspended in 100 μL of chilled 1 x cell lysis buffer, incubated on ice for 10 minutes followed by centrifugation. Equal amounts of supernatant protein were used in the absence or presence of caspase-8 or caspase-9 inhibitors. Lysates were preincubated with caspase inhibitors for 10 minutes before adding caspase-8 or caspase-9 inhibitor substrate solution. All samples were incubated for 90 minutes at 37°C and then read at 405 nm in a microtiter plate reader. Fold increase in caspase-8 or -9 activity was determined by comparing the optical density (OD) reading from the treated sample with the level of the untreated control.

Determination of mitochondrial DNA damage
Quantitative alkaline Southern blotting was conducted as described previously (13). Following treatment, DNA was isolated and digested with BamHI. Sodium hydroxide (NaOH) was included in the loading dye, agarose gel, and electrophoresis buffers. The ethidium bromide–stained gel served as an internal control for equal loading between the lanes. After blotting BamHI-digested total DNA, the membrane was hybridized with a mitochondrial-specific probe, which recognizes the CoI-ATPase 6 sequences; this probe recognizes a major BamHI restriction band that is larger than the sequence of the probe and allows for analysis of damage over a longer stretch of DNA than just the CoI-ATPase 6 sequence. After hybridization, membranes were exposed to an imaging screen to determine band intensity. A decrease in the hybridization of the mitochondrial-specific probe correlates with increased DNA damage. The number of pixels per band was determined by encompassing bands with identical rectangular regions of interest and subtracting the background. The break frequency was determined using the Poisson expression (s = −ln P0), where s is the number of breaks per fragment and P0 is the fraction of fragments free of breaks.

Statistical analysis
All numerical data are represented as the mean and SD of triplicate data points for each group. Statistical analyses were conducted using Student t test and P < 0.05 was considered statistically significant.

Results
Generation and characterization of human myeloid precursor cells
Human CD34+ cells were incubated with human G-CSF and SCF to generate large numbers of myeloid precursor cells so that sufficient amounts of DNA, RNA, and protein could be obtained for analysis. By days 10 to 12 postexpansion, myeloid precursor cells represented the majority of the cell population (see Supplementary Fig. S1).

Induction of DNA-damage response following exposure to myelosuppressive doses of chemotherapy
Myeloid precursor cells were exposed to 200 μmol/L of TMZ, which, based on pharmacokinetic studies in patients, would most likely exceed the maximal tolerated dose (MTD) of TMZ, and therefore potentially lead to a myelosuppressive or myeloablative condition. For example, in a phase I trial in which submyelosuppressive doses of TMZ were used, a single dose of TMZ at 200 mg/m² resulted in peak TMZ levels (Cmax) in the plasma of approximately 11 μg/mL or 57 μmol/L; when TMZ was administered daily for 5 consecutive days, peak levels of TMZ in the plasma were approximately 16 μg/mL or 82 μmol/L (14). In addition, Quinn and colleagues conducted a phase I clinical trial in adult patients with malignant glioma to determine the MTD of single-dose TMZ in combination with 6BG. When combined with 6BG, the MTD of TMZ was found to be 472 mg/m² (6). The plasma levels associated with a dose of TMZ at 472 mg/m² have been reported in a phase I pediatric trial; the range of peak TMZ levels was 0.63 to 2.65 μg/mL or approximately 3 to 136 μmol/L in the plasma (15).

To understand how chemotherapy-mediated myelosuppression occurs when using 6BG in combination with TMZ, we first examined the impact of 6BG/TMZ exposure in myeloid precursor cell viability assays. MGMT-mediated repair of TMZ-induced adducts is initiated by the transfer of the methyl group from the O6 position of guanine to an internal cysteine residue in the active site of MGMT, which results in ubiquitination and proteosome-mediated degradation of MGMT. In the presence of the MGMT inhibitor 6BG, MGMT is subsequently ubiquitinated and degraded because of transfer of the benzyl group from 6BG to the same cysteine residue in the active site (9). 6BG/TMZ–based regimens have been used to downregulate MGMT expression in tumors and increase sensitivity to TMZ (9). Myeloid precursor cells were exposed to 6BG/TMZ one time and compounds were not washed out. As expected, exposure of
myeloid precursor cells to 6BG and/or TMZ resulted in decreased MGMT levels (Fig. 1A) by day 1 postexposure. Consistent with depletion of MGMT, time-course viability studies of myeloid precursor cells indicated that the combination of 6BG and TMZ resulted in a significant increase in cell death by day 4. By day 7 posttreatment, 90% of the 6BG/TMZ–treated cells were nonviable (Fig. 1B). In TMZ-treated cultures, approximately 40% of the cells were nonviable by day 7 posttreatment. In the control and 6BG-treated cultures, cell death increased from approximately 10% to 20% to 25% over time, which is typically seen in this culture system as the population moves toward a more differentiated phenotype.

To delineate signaling pathways operative following DNA damage, gene expression was analyzed in myeloid precursor cells treated with 6BG/TMZ for 18 hours. Microarray analysis was conducted on treated myeloid precursor cells using the PIQOR Cell-Death microarray, which contains 494 probes specific for RNAs whose protein products are involved in apoptosis and include stress-like caspases, TNF-receptor family members, Bcl-2 family members, and HSPs. Most of the increased RNA transcripts were targets of p53-mediated signaling. The 6BG/TMZ combination had the strongest impact on changes in gene expression in the myeloid precursor cells compared with control, 6BG alone, or TMZ alone (see Supplementary Table S1). The data

![Graphical representation of Figure 1](image_url)

**Figure 1.** Correlation of MGMT status, cell viability, and DNA damage response in myeloid precursor cells following exposure to 6BG/TMZ. A, human myeloid precursor cells were exposed to 6BG in the absence or presence of TMZ (200 and 400 μmol/L) and 6BG/TMZ for 1 day and probed for MGMT expression. β-Actin served as loading. B, myeloid precursor cells were treated in triplicate and viability of myeloid cultures determined over time. C-F, analysis of 6BG/TMZ–induced p53 stabilization, p21, AKT phosphorylation, and cell-cycle arrest at day 3 posttreatment. GAPDH served as a loading control. *, P < 0.05, 6BG/TMZ versus control, 6BG, or TMZ. Data are representative of 3 independent experiments.
discussed in this publication have been deposited in National Center for Biotechnology Information’s (NCBI) Gene Expression Omnibus (GEO; ref. 16) and are accessible through GEO Series accession number GSE44122 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44122). On the basis of the microarray data obtained from the myeloid precursor cells, protein expression was evaluated on the basis of selected RNAs that were increased (p21 and FAS) or showed no change (p53) following 6BG/TMZ treatment. TMZ and 6BG/TMZ exposure resulted in a stabilization of p53 (Fig. 1C), and increased levels of the p53-downstream target p21 (Fig. 1D). Exposure of myeloid precursor cells to 6BG/TMZ resulted in no changes in total AKT protein but decreased phosphorylation of AKT at serine 473 compared with control, 6BG, or TMZ (Fig. 1E). Decreased phosphorylation of AKT at serine 473 has been shown previously to correlate with decreased AKT activity (17). These DNA-damage responses correlated with increased cell-cycle arrest in G0–G1 and G2–M; the sub-G0–G1 fraction also increased significantly by day 3 posttreatment with 6BG/TMZ (Fig. 1F).

**Myelosuppressive chemotherapy-mediated DNA damage and cell death**

Although the nuclear genome is commonly examined for DNA damage, the mitochondrial genome plays an essential role in regulating cell survival, as this genome encodes for essential proteins involved in oxidative phosphorylation and respiration. Moreover, the extent to which myelosuppressive chemotherapy actually damages the mitochondrial genome of hematopoietic cells, and how damage correlates with cell death, is not known. The degree of mitochondrial-specific DNA-strand breaks was determined in myeloid precursor cells (control, 6BG, TMZ, and 6BG/TMZ) by alkaline Southern blot analysis using a mitochondrial-specific Coll-ATPase 6 DNA probe (13). The lack of hybridization to the mitochondrial-specific probe is indicative of mitochondrial DNA damage. In cultures exposed to 6BG, there was no increase in mitochondrial DNA damage (Fig. 2A). When myeloid cells were exposed to increasing doses of TMZ in the presence of 6BG, there was a dramatic increase in the number of mitochondrial DNA-strand breaks compared with TMZ alone, indicating that the rate of repair was diminished in the 6BG/TMZ-treated cultures (Fig. 2A). The increase in mitochondrial DNA damage correlated with doses of 6BG/TMZ resulting in cell death by day 4 posttreatment as shown in Fig. 1B. The levels of γ-H2AX that are indicative of double-strand DNA-strand breaks were increased in total cellular lysates following 6BG/TMZ treatment (Fig. 2B). By day 4 posttreatment with 6BG/TMZ, mitochondrial membrane depolarization (Fig. 3A) and apoptotic cells were increased in cultures compared with TMZ or control alone (Fig. 3B). To examine if the intrinsic pathway is activated in response to 6BG/TMZ, we evaluated caspase-9 activity following treatment. Levels of activated caspase-9 activity increased significantly following exposure to 6BG/TMZ (Fig. 3C) and correlated with activation of caspase-3 and -7 (Fig. 3D), indicating that mitochondrial-mediated cell death is activated and plays a prominent role in chemotherapy-mediated myeloid cell death.

**Lack of FAS-mediated signaling during exposure of cells to myelosuppressive chemotherapy**

Time-course studies indicated that FAS expression increased as the human CD34⁺ cells were pushed via cytokine exposure to proliferate and differentiate into myeloid precursor cells (Fig. 4A). The percentage of FAS⁺ myeloid precursor cells as well as the density of FAS per cell increased following 6BG/TMZ treatment compared...
with control, 6BG, or TMZ treatment (Fig. 4B). FAS-mediated signaling typically results in activation of caspase-8 and -9 leading to subsequent apoptosis. To investigate the relevance of FAS-mediated signaling in more detail, the levels of c-Flip<sub>long</sub> and c-Flip<sub>short</sub> in myeloid precursor cells were determined as both c-Flip isoforms can inhibit FAS-mediated signaling in other cell systems (18, 19). Both c-Flip<sub>long</sub> and c-Flip<sub>short</sub> were expressed in myeloid precursor cells. No changes in the level of c-Flip<sub>long</sub> were observed following treatment with 6BG alone, TMZ alone, or 6BG/TMZ (Fig. 4C). However, in comparison with control cells or cells treated with 6BG or TMZ, there was a decrease in c-Flip<sub>short</sub> in myeloid precursor cells exposed to 6BG/TMZ. We next determined if decreases in c-Flip<sub>short</sub> correlated with activation of FAS-mediated signaling (Fig. 5). To directly determine if FAS-mediated signaling is operative during 6BG/TMZ–mediated cell death, agonistic (CH-11) and neutralizing (ZB4) anti-FAS antibodies were used. Jurkat cells served as a positive control as FAS-mediated signaling is operative when these cells undergo apoptosis (20). Treatment of Jurkat cells with agonistic anti-FAS antibodies resulted in cell death. This could be prevented by preincubation of Jurkat cells with the neutralizing anti-FAS antibodies followed by incubation with the agonistic anti-FAS antibodies (Fig. 5A). When 6BG/TMZ–treated myeloid precursor cells were incubated with agonistic anti-FAS in the absence or presence of isotype control or neutralizing anti-FAS antibodies for 3 days, there were no significant changes in cell death, indicating that at least under these conditions, FAS-mediated signaling was not operative in myeloid precursor cells treated with 6BG/TMZ. Caspase-8 has been previously reported to be involved in myeloid cell differentiation (21, 22) and bioactivity analyses indicated detectable levels of caspase-8 activity in the myeloid cultures; however, the levels of active caspase-8 did not change following exposure to 6BG/TMZ (Fig. 5B). These data show that while the extrinsic, FAS-mediated cell-death pathway is upregulated molecularly in response to 6BG/TMZ treatment, it is the intrinsic, mitochondrial-mediated pathway that is fully activated and correlates with increased apoptosis of myeloid precursor cells.

Discussion

In the present myeloid precursor ex vivo model, we determined the extent to which the mitochondrial- versus FAS-mediated cell-death pathways are activated in 6BG/TMZ–treated myeloid precursor cells. We focused on DNA-damage responses elicited via a TMZ-based regimen previously used in the clinic that can be myelosuppressive (6). In 6BG/TMZ–treated myeloid precursor cells, p53-mediated signaling was activated and the intrinsic, mitochondrial-mediated cell death pathway predominated following exposure to 6BG/TMZ. Interestingly, damage to both mitochondrial and nuclear DNA persisted following exposure to 6BG/TMZ. Increases in mitochondrial membrane depolarization and caspase-9 activity following 6BG/TMZ exposure were...
observed. Decreased phosphorylation of AKT at serine 473, an indicator of decreased AKT activity, was observed in 6BG/TMZ–treated myeloid precursor cells (23). AKT blocks apoptosis through phosphorylation and inactivation of proapoptotic proteins such as Bad (24), forkhead transcription factors (25), and caspase-9 (26). Decreased AKT activity correlated with increased caspase-9 activity in treated myeloid precursor cells. In comparison with control, 6BG, or TMZ, cell-surface FAS expression increased in 6BG/TMZ–treated myeloid precursor cells. Caspase-8 inhibitors, c-FLIPlong and c-FLIPshort, which can associate with the FAS-receptor complex were constitutively expressed in myeloid precursor cells, and c-FLIPshort expression decreased in the presence of 6BG/TMZ. However, this did not correlate with increases in caspase-8 activity. In addition, experiments with agonistic and neutralizing anti-FAS antibodies further confirmed that FAS-mediated signaling resulting in cell death was not operative.

The response of mammalian cells to DNA-damaging agents is dependent on complex intracellular signaling networks and the interplay of these networks can be cell-type specific. Depending on the cell type studied, the

Figure 4. Modulation of FAS and c-FLIP expression in myeloid precursor cells exposed to 6BG/TMZ. A, time course of FAS expression was conducted during the expansion period. FAS expression was analyzed in triplicate via flow cytometry on cells in the early expansion and differentiation phase—days 0 to 8 postexpansion. B, at 10 days postexpansion, control myeloid precursor cells (C) or myeloid precursor cells were exposed to 6BG, TMZ, or combination of 6BG/TMZ for 3 days. The percentage of FAS-positive and the relative density of FAS expression was determined via flow cytometry. C, myeloid precursor cells were exposed to 6BG, TMZ, or 6BG/TMZ for 3 days and cellular lysates analyzed via Western blot analysis for c-FLIPshort and c-FLIPlong. Flow-cytometric data are expressed as the mean and SD of triplicate data points for each group. Flow-cytometric data and Western blot analysis were repeated twice with similar results.

Figure 5. Relevance of FAS-mediated signaling in myeloid precursor cells exposed to 6BG/TMZ. A, as a control for antibody-mediated signaling through FAS, Jurkat T cells were incubated in triplicate with agonistic (Ag), neutralizing (Neut), isotype (Iso), or combinations of antibodies for 24 hours, and the percentage of nonviable cells determined via Annexin V staining and flow cytometry. Myeloid precursor cells that had been expanded in culture for 10 days were treated with 6BG/TMZ for 3 days in the presence of combinations of agonistic (Ag), neutralizing (Neut), and isotype (Iso) antibodies. Antibody was added each day of the 3-day culture and nonviable cells determined via Annexin V staining and flow cytometry on day 4 posttreatment. B, caspase-8 (C-8) activity was determined in lysates from control and 6BG/TMZ–treated cells in the presence of a C-8–specific inhibitor. Data are representative of 2 independent experiments (A) or 3 independent experiments (B).
extrinsic, death receptor–mediated versus intrinsic, mitochondrial-mediated apoptotic pathways induced by chemotherapy can be preferentially activated (27–30). Although O6MeG adducts induce FAS-mediated apoptosis in primary human T-lymphocytes (29), formation of O6MeG adducts induced mitochondrial-mediated cell death in primary human fibroblasts (28) and B-lymphoblastoid cells (27). In glioblastoma multiforme cells, Roos and colleagues previously showed that relative rates of cell proliferation, double-strand breaks, MGMT levels, and p53 status could dictate whether GBM cells died predominantly via a mitochondrial- versus FAS-mediated apoptotic cell death (30).

In myeloid precursor cells, although FAS was expressed and c-FLIP_{short} levels were decreased in 6BG/TMZ-treated cells, myeloid precursor cells were not sensitive to signals delivered through the FAS receptor complex. There are 3 isoforms of c-FLIP that can be detected in mammalian cells—c-FLIP_{long}, c-FLIP_{RaJI (R)}, and c-FLIP_{short} (19). Both the c-FLIP_{long} and c-FLIP_{short} were constitutively expressed on the myeloid precursor cell, and only the c-FLIP_{short} isoform was modulated by 6BG/TMZ exposure. Others have shown that both of these isoforms can be found at the FAS death-inducing signaling complex (DISC) and can block procaspase-8 activation and death receptor–mediated apoptosis (19). However, the precise function of c-FLIP_{long}—proapoptotic versus antiapoptotic can be based on cellular context. For example, c-FLIP_{long} exhibits proapoptotic function when there is strong receptor activation or when high levels of short c-FLIP isoforms—c-FLIP_{short} or c-FLIP_{RaJI}—are present (31). Poukoula and colleagues previously showed that c-FLIP_{short} in contrast to c-FLIP_{long} was more susceptible to ubiquitination and proteasomal degradation and was partially attributable to 2 lysine residues in the C-terminal 20 amino acids that are unique to c-FLIP_{short} (32). Whether FAS-mediated signaling plays a different functional role or becomes active once the myeloid precursor cells fully mature, is possible. Bauer and colleagues recently reported that TMZ-mediated apoptosis of human peripheral blood monocytes induced both the mitochondrial- and FAS-mediated apoptotic pathways (33).

Maintenance of both nuclear and mitochondrial genome stability is essential for normal cell survival and also prevents the emergence of precancerous cells. Mitochondria function not only as the primary energy producer but also as a key regulator of cell survival and death (34). Other laboratories have shown that mitochondrial DNA can incur greater levels of damage than nuclear DNA following treatment with alkylating or oxidizing agents (35–37). Indeed, various disease states such as diabetes, ischemic heart disease, Parkinson’s disease, Alzheimer’s disease, and the normal aging process have been correlated with increased mutations in mitochondrial DNA (38, 39). Studies of mitochondrial DNA repair show the presence of base excision repair (39, 40) and MMR in the mitochondria (41, 42) but no nuclear excision repair. There is some evidence for reversal of O6-methylguanine lesions by direct repair in mitochondria (43, 44). However, MGMT is most likely not found in the mitochondria as it does not contain a mitochondrial-localization signal. In addition, we previously could not detect endogenous MGMT protein in the mitochondria via confocal microscopy in human hematopoietic cells (45). The importance of mitochondrial DNA damage was previously documented by our group. In this study, we showed that forced localization of MGMT via fusion of a mitochondrial-localization signal to MGMT, resulted in detectable MGMT in the mitochondria and that expression of MGMT in the mitochondria correlated with increased resistance to alkylator exposure in primary hematopoietic progenitor cells and in the leukemic cell line K562 (45). In our current study, we directly measure DNA damage and repair kinetics in the mitochondria of myeloid precursor cells. Significant levels of DNA damage in the mitochondrial genome of 6BG/TMZ–treated myeloid precursor cells were evident. We also show that depletion of a DNA repair protein (i.e., MGMT) that is normally only localized in the nucleus can have a profound effect on mitochondrial DNA damage status and function. In addition, lack of repair to the mitochondrial DNA can lead to a secondary generation of ROS, which also triggers the intrinsic pathway of apoptosis.

An inverse correlation existed between mitochondrial DNA damage and the presence of MGMT in myeloid precursor cells. A tight interconnection between mitochondrial and nuclear genome integrity most likely accounts for our observations. The persistence of nuclear DNA damage caused by 6BG-mediated MGMT downregulation and activation of p53-mediated signaling is most likely linked to mitochondrial dysfunction. Furthermore, a large number of genes encoding mitochondrial-localized proteins are found in the nuclear genome and damage to the nuclear genome could affect transcription of numerous nuclear-encoded genes (46). In addition, increases in oxidative damage, abasic sites, methylated adducts, and DNA-strand breaks in the mitochondrial DNA could all lead to mitochondrial dysfunction and apoptosis. Consistent with a close relationship between integrity of mitochondrial and nuclear genomes, Martin and colleagues previously showed that the PTEN-induced putative kinase 1 (PINK1), a nuclear-encoded protein that is specifically found in the mitochondria, has a profound effect on nuclear DNA integrity (47). In this study, both nuclear and mitochondrial oxidative DNA lesions were enhanced in cells that did not express mitochondrial-localized PINK1. These findings suggest that modulation of mitochondrial-specific DNA damage could affect nuclear-specific DNA damage and that regulation of mitochondrial and nuclear integrity can be closely linked. Our study is the first to document the presence of mitochondrial DNA damage following exposure to TMZ and 6BG/TMZ in primary human myeloid precursor cells. In addition, we found that the degree of nuclear DNA damage in myeloid precursor cells depleted of MGMT via 6BG could influence mitochondrial function.

Basic research investigations into understanding how nontransformed cells respond to and repair promutagenic DNA lesions will be important for future screening and
refinement of new therapeutics. Adequate detection of intracellular signaling proteins and modulation of these pathways can be challenging when working with primary culture systems. In this model system, generation of primary human myeloid precursor cells required only 2 cytokines: G-CSF and SCF. Large numbers of cells adequate for most molecular analyses could be obtained. Different hematopoietic lineages can have differential sensitivities to genotoxic agents. For example, others have shown that human monocytes not only macrophages or monocyte-derived dendritic cells were highly sensitive to the killing effect of TMZ and other methylating agents but had similar sensitivities to the cross-linking chemotherapeutics fotemustine, mafosfamide, and cisplatin (33, 48). Although hematopoietic CFU assays (49) and humanized mouse models (11) have merit as screening tools for compound-induced toxicity, myeloid precursor cells can be used not only for screening new therapeutics but also for comparing the pharmacodynamic molecular profiles of how normal hematopoietic versus cancer cells respond to different chemotherapeutic compounds. It is important to emphasize, however, that alkylating agents such as TMZ can be toxic to hematopoietic stem cells and toxicity can be more pronounced when given in combination with 6BG. Although the myeloid precursor model cannot be used to probe mechanisms of alkylating agent-mediated toxicity to the hematopoietic stem-cell compartment, it does present a feasible in vitro model to understand how primary myeloid cells respond at the molecular level to therapy-induced DNA damage. Because of differences in cell cycling, DNA repair capacity, and bone marrow microenvironmental cues, it is highly likely that the kinetics and molecular mechanisms of DNA damage responses in progenitors versus stem cells are different. For example, in studies by Milavsky and colleagues, human hematopoietic stem cells derived from UCB exhibited slower repair of ionizing radiation-induced DNA damage compared with progenitor cells; this correlated with increased apoptosis of stem cells compared with the progenitor cells (50). It would be of interest in the future studies to investigate molecular responses of nucleotide analogs such as cytarabine and vidarabine that specifically target more rapidly cycling hematopoietic progenitor cells, as studied in the present model. The myeloid precursor cell model can also be used to explore at the molecular level the sensitivity to different modes of DNA damage, signaling mechanisms operative under different types of DNA stress, and for testing strategies that improve DNA repair and maintain genome integrity in normal cells.

Disclosure of Potential Conflicts of Interest

W.S. Goebel is employed as Medical Director in Cook General Biotech-

tology, LLC. No potential conflicts of interest were disclosed by the other authors.

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References


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