DNA Mismatch Repair (MMR) Mediates 6-Thioguanine Genotoxicity by Introducing Single-strand Breaks to Signal a G_2-M Arrest in MMR-proficient RKO Cells

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ABSTRACT

Purpose: The DNA mismatch repair (MMR) system plays an important role in mediating cell death after treatment with various types of chemotherapeutic agents, although the molecular mechanisms are not well understood. In this study, we sought to determine what signal is introduced by MMR after 6-thioguanine (6-TG) treatment to signal a G_2-M arrest leading to cell death.

Experimental Design: A comparison study was carried out using an isogenic MMR^+ and MMR^- human colorectal cancer RKO cell system, which we established for this study. Cells were exposed to 6-TG (3 \mu M \times 24 h) and then harvested daily for the next 3–6 days for growth inhibition assays. Cell cycle effects were determined by flow cytometry, and DNA strand breaks were measured using pulsed-field gel electrophoresis and alkaline Comet assays.

Results: We first established MMR^+ RKO cell lines by transfection of human MutL homologue 1 (hMLH1) cDNA into the hMLH1-deficient (MMR^-) RKO cell line. The ectopically expressed hMLH1 protein restored a MMR-proficient phenotype in the hMLH1^+ transfectants, showing a significantly increased and prolonged G_2-M arrest followed by cell death after 6-TG exposure, compared with the vector controls. The MMR-mediated, 6-TG-induced G_2-M arrest started on day 1, peaked on day 3, and persisted to day 6 after 6-TG removal. We found that DNA double-strand breaks were comparably produced in both our MMR^+ and MMR^- cells, peaking within 1 day of 6-TG treatment. In contrast, single-strand breaks (SSBs) were more frequent and longer lived in MMR^- cells, and the duration of SSB formation was temporally correlated with the time course of 6-TG-induced G_2-M arrest.

Conclusions: Our data suggest that MMR mediates 6-TG-induced G_2-M arrest by introducing SSBs to signal a persistent G_2-M arrest leading to enhanced cell death.

INTRODUCTION

The human MMR system is an important DNA repair pathway, which serves as a caretaker of genome integrity. MMR corrects mismatched nucleotides and insertion-deletion loops generated by DNA replication errors, with MMR-deficient (MMR^-) cells demonstrating increased genomic instability (1–4). The function of human MMR is carried out by multiple MMR protein heterodimers. Repair is initiated by recognition and binding of hMutS{alpha} (a heterodimer of MMR proteins hMLH2/hMSH6) or hMutS{beta} (hMLH2/hMSH3) to the mismatch site, followed by formation of a tetramer repair complex of hMutL{alpha} (hMLH1/hPMS2) or hMutL{beta} (hMLH1/hMLH3) with either hMutS{alpha} or hMutS{beta} (1, 4). The downstream repair proteins are then recruited to the sites to complete the repair process by the sequential steps of incision, excision, synthesis, and ligation, although the details of these downstream repair processes are not clear. hMLH1 and hMSH2 proteins appear to play a key role in MMR function because a majority of hereditary nonpolyposis colon cancer cases are associated with defects in either hMLH1 or hMSH2 protein expression and loss of MMR function (5). Loss of hMLH1 or hMSH2 protein expression also occurs in several different types of sporadic cancers including colorectal, gastric, and endometrial carcinomas and is typically associated with the microsatellite instability high phenotype (6–13).

Although the primary function of MMR is to edit DNA replication errors, its involvement in cellular responses to a variety of chemotherapeutic drugs has been recently demonstrated. MMR-proficient (MMR^+) cells have been shown to be sensitive to killing by 6-TG, N-methyl-N'-nitro-N-nitrosoguanidine, methyltritosuroea, temozolomide, Adriamycin, procabazine, busulfan, VP-16 (etoposide), cisplatin, carboplatin, benzo(a)pyrene, 5-fluorouracil, and 5'-deoxy-5-fluorouridine (14–17). In contrast, MMR^- cells show variable levels of in vitro and in vivo resistance to these drugs. Additionally, there are evolving clinical data suggesting a “drug-resistance” phenotype in MMR^-/microsatellite instability high human tumors (18, 19). In this scenario, functional MMR does not enhance cell survival but rather introduces persistent signals, which trigger a...
prolonged G2-M arrest leading to cell death. However, the molecular mechanisms of the MMR-mediated drug cytotoxicity are not yet precisely elucidated. Little is known about what signals are generated by MMR after drug treatment. Two models have been proposed: (a) a futile repair cycle model; and (b) a general DNA damage sensor model (20). In the futile repair cycle model (21–23), MMR attempts to process drug-induced DNA lesions in the daughter strands without removing damage from the parental strands, which is thought to lead repetitive strand breaks that may be a signal to a G2-M arrest. In the general DNA damage sensor model (24, 25), the assembly of MMR proteins, possibly together with other repair-related proteins, at the drug-induced lesions makes a signal. DNA strand breaks are not believed to be a prerequisite for G2-M arrest signaling in this second model. However, direct evidence supporting either of these models of MMR-mediated, drug-induced G2-M arrest is lacking.

In this study, we used the chemotherapeutic drug 6-TG to test the futile repair cycle model and to determine whether DNA DSBs or SSBs result from MMR processing after 6-TG treatment. 6-TG is a purine antimetabolite that has been in clinical use as an antileukemic agent for many years. 6-TG is incorporated into DNA by mammalian cells in place of dGTP during the course of replication, and a small amount (~1 in 106 bases) of incorporated 6-TG is then methylated in situ to 6-meTG by endogenous 5-adenosymethyltransferine (26). During the next replication cycle, 6-meTG can pair with either a cytosine or a thymine base. Both 6-meTG-cytosine and 6-meTG-thymine mismatches are recognized by MMR (26–28). MMR cells are highly sensitive to 6-TG, showing an initial prolonged G2-M arrest followed by cell death.

Another goal of this study was to establish a more defined MMR+ human tumor cell model through transfection of only hMLH1 cDNA into a hMLH1− human tumor cell line. We recognized that the available hMLH1 human tumor cell model systems used previously by our group and others involved a transfer of a single entire human chromosome 3 into the hMLH1− human colorectal carcinoma cell line HCT116 (29) or the human ovarian carcinoma cell line A2780/CP70 (30). However, chromosome 3 also encodes other important genes that may interact with MMR processing including the DNA repair-related glycosylases 8-oxoguanosine DNA glycosylase and methyl-CpG binding endonuclease 1, the DNA damage checkpoint pathway kinase ataxia-telangiectasia and Rad3-related, methyl-CpG binding endonuclease 1, the DNA damage checkpoint kinase ataxia-telangiectasia and Rad3-related, methyl-CpG binding endonuclease 1, the DNA damage checkpoint kinase ataxia-telangiectasia and Rad3-related, methyl-CpG binding endonuclease 1, the DNA damage checkpoint kinase ataxia-telangiectasia and Rad3-related.

In this study, we established a MMR+ cell model by transfection of hMLH1 cDNA into a hMLH1− RKO (human colon cancer) cell line. We then used this isogenic RKO cell system to study the temporal relationships of 6-TG cytotoxicity and cell cycle changes to the formation of DNA DSBs and SSBs in MMR+ and MMR− cells after 6-TG treatment. Treatment with 6-TG results in significantly increased cytotoxicity in MMR− RKO cells versus MMR− RKO cells using both short-term (growth inhibition) and long-term (clonogenic survival) assays. Our data show that in response to 6-TG, DSBs are comparably produced in both our MMR+ and MMR− cells, peaking within 1 day after 6-TG treatment, whereas SSBs are more frequent and longer lived in MMR+ cells for 3 or more days after drug exposure. The persistent SSB in MMR− RKO cells correlated temporally to the G2-M arrest after 6-TG treatment, suggesting that SSBs may be a signal for subsequent MMR-mediated, 6-TG-induced G2-M arrest and cell death.

**MATERIALS AND METHODS**

**Cell Culture and cDNA Transfection.** RKO is a human colorectal carcinoma cell line deficient in hMLH1 because of hypermethylation of the gene promoter region (31). HT29, a human colorectal carcinoma cell line proficient in MMR, was used as a control for hMLH1 and hMSH2 protein expression. Cells were grown in DMEM (Mediatech, Herndon, VA), supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM glutamine, and 0.1 mM nonessential amino acids (Life Technologies, Inc., Gaithersburg, MD) in 10% CO2 at 37°C.

The RKO cells were subcloned before transfection, and a single clone was chosen as a parental cell line (doubling time = 18 ± 2.1 h). A full-length sense hMLH1 cDNA Xhol fragment (2484 bp; Ref. 32) was constructed into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) under the control of a cytomegalovirus promoter. The transfection was performed with LipofectAMINE (Life Technologies, Inc.), according to the manufacturer’s recommendations. The clones were selected with 500 μg/ml G418 (Life Technologies, Inc.) for 2 weeks and screened by Western blotting for hMLH1-expressing clones. An empty vector was transfected in a similar fashion, and clones were selected to serve as controls.

**PCR Analysis.** The exogenous hMLH1 cDNA was confirmed using PCR. Cell genomic DNA was isolated using DNAzol (Molecular Research Center, Inc., Cincinnati, OH). The sequence of the forward primer (T7 promoter sequence) was 5′-TAATACGACTCACTATAGGG-3′, and the sequence of the reverse primer (hMLH1 exon 1 sequence) was 5′-CTCGTC-CAGCCGCAATAAA-3′. PCR was performed in a final volume of 50 μl containing 200 ng of genomic DNA, 1.5 μM of each primer, 0.2 mM of each deoxynucleoside triphosphate, 1.5 mM MgCl2, and 1.25 units of Taq DNA polymerase (MBI Fermentas, Hanover, MD). After denaturation for 2 min at 94°C, the PCR consisted of 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by a final extension for 7 min at 72°C. The PCR products were examined on 2% agarose gel.

**Antibodies.** The antibodies used in this study were as follows: anti-hMLH1 and anti-hPMS2 (BD PharMingen, San Diego, CA); anti-hMSH2 (Calbiochem, San Diego, CA); anti-actin (Sigma, St. Louis, MO); and secondary antibody IgG-horseradish peroxidase conjugates (Santa Cruz Biotechnology, Santa Cruz, CA).

**Western Blotting Analyses and Clonogenic Survival Assays.** Western blotting analyses were carried out as described previously (33). Clonogenic survival assays after treatment with 6-TG were performed as follows. Cells were seeded at 2.5 × 106 cells/10-cm dish in 10 ml of the complete medium and allowed to adhere for ~15 h. 6-TG at different doses (0–3 μM) was then added to the medium for 24 h, and, subsequently, the
cells were reseeded into 60-mm dishes with 5 ml of drug-free medium and allowed to grow for ~15 days for colony formation. The colonies were stained with 0.5% crystal violet in methanol/acetic acid (3:1), and those colonies of 50 or more cells were counted. Experiments were performed in medium without G418, and all experiments were performed twice, each in triplicate.

**Schedule of 6-TG Treatment for Cell Cycle Analysis.**

Cells were seeded at 2.5 × 10^5 cells/10-cm dish and allowed to attach and grow for ~15 h. 6-TG (0 or 3 μM) was then added to the medium at this time (t = day −1). After 24 h, 6-TG was removed (t = day 0), and drug-free medium was added. The treated (3 μM 6-TG) and control (0 μM 6-TG) cells were then harvested daily for the next 3 days (t = days 1–3) for cell cycle analysis and for the next 6 days (t = days 1–6) for cell number counting. These experiments were performed three times for both flow cytometric and cell number analyses.

**Flow Cytometry Analysis.** To determine cell cycle changes after 6-TG exposure, cells were handled according to the treatment schedule mentioned above. Cells were harvested immediately after 6-TG treatment (day 0) and then daily for 3 days and fixed in 95% ethanol. We also treated cells with IR or VP-16 as other DNA-damaging agents to assess cell cycle changes. For IR or VP-16 experiments, cells were seeded at 2.5 × 10^5 cells/10-cm plate in 10 ml of medium and irradiated ~15 h later with a Model 109 137Cs irradiator to 6 Gy at a dose rate of 4.0 Gy/min or treated with 5 μM VP-16 for 2 h. The cells were then harvested at earlier time points (0–12 h after IR or 0–10 h after VP-16 treatment) and fixed as described above. For PI staining, samples were washed with PBS, resuspended in PI solution (1 mg/ml RNase A, 33 μg PI/ml in PBS, 0.5 mM EDTA, and 0.2% NP40), and then incubated at room temperature for 30 min. Flow cytometry was performed using a Coulter EPICS XL-MCL flow cytometer (Coulter Corp., Miami, FL). Flow cytometry data were analyzed for cell cycle profiles using ModFit LT version 3.0 software (Verity Software, Topsham, ME). All experiments were repeated at least three times.

**PFGE.** Cells treated with 6-TG (1.5–6 μM for 24 h) or IR (6 Gy) were trypsinized, counted, and resuspended in PBS at a concentration of 2 × 10^6 cells/ml. The cells were then mixed (1:1) with 1.5% low melting point agarose (BioWhittaker Molecular Applications, Rockland, ME), and 80 μl of the mixture were loaded into wells of the plug mold (Bio-Rad Laboratories, Hercules, CA). The plugs were allowed to solidify at 4°C for 10 min and then placed into tubes containing 0.4 ml/plug lysis buffer [10 mM Tris (pH 7.8), 50 mM NaCl, 100 mM EDTA, 1% Sarkosyl, and 1 mg/ml proteinase K] and incubated at 50°C overnight. The plugs were then washed five times with washing buffer [20 mM Tris and 50 mM EDTA (pH 8.0)] for 5 min. The electrophoresis was performed using a 0.7% agarose gel with 1 × TAE buffer [40 mM Tris-acetate/1 mM EDTA (pH 8.0)] with a CHEF-DR III system (Bio-Rad Laboratories). The conditions for electrophoresis were as follows: for block 1, switch time was 1500 s for 12 h at 2.0 V/cm using an included angle of 106°; for block 2, the switch time was 1800 s for 24 h at 2.0 V/cm using an included angle of 106°. Gel images were obtained by photographing ethidium bromide-stained gels under UV light and quantitated with ImageJ software (NIH, Bethesda, MD) for fluorescence density.

**Alkaline Comet Assay.** The alkaline Comet assay was carried out using a CometAssay Kit (Trevigen, Gaithersburg, MD), according to the manufacturer’s procedures. Briefly, 6-TG (3 μM × 24 h)-treated cells or control cells were scraped, counted, and diluted to 2 × 10^5 cells/ml in PBS. Cells were then mixed with low melting point agarose (1:10), and 75 μl of the mixture were then placed on a CometSlide (Trevigen). After the gel was solidified at 4°C, the slide was incubated with lysis solution at 4°C for 60 min. The slide was then transferred to an alkaline solution at room temperature for another 20 min to allow DNA unwinding. The alkaline electrophoresis was carried out with 1 V/cm and 300 mA at 4°C for 40 min. The same electrophoresis unit and power supply were used throughout the study. All procedures were conducted under dimmed light. The slides were then dipped in 70% ethanol for 5 min and allowed to air dry. The DNA was stained with Hoechst 33342 (Molecular Probes, Eugene, OR) and visualized using a fluorescence microscope. The Comet image was analyzed with ImageJ software for tail moment (fraction of DNA in the tail × tail length), and 50 Comets were measured for each sample.

**Statistics.** The data, where applicable, represent means ± SE. Data were analyzed using the Student t test.

**RESULTS**

**Stable Expression of the hMLH1 cDNA Restores a MMR+ Phenotype in RKO Cells.** Considering possible genotypic heterogeneity within a MMR- human tumor cell population because of its recognized high spontaneous mutation rate (34), we initially subcloned RKO cells, selecting one clone as a parental cell line (RKO-p) to start the transfection experiment. The hMLH1 cDNA was then stably transfected into the cloned (RKO-p) cell population, and two hMLH1-expressing clones were selected (M1 and M4). As a control, the empty vector was also stably transfected into RKO-p cells, and again two clones were selected (V1 and V2). As shown in Fig. 1A, the M1 and M4 cell lines expressed hMLH1 protein, at levels slightly lower than that of endogenous hMLH1 protein as found in the MMR- human colorectal tumor cell line HT29 (35). Another important MMR protein, hMSH2, was also examined, and its level did not change in the hMLH1- transfectants (Fig. 1A). hPMS2, a dimeric partner of hMLH1, could also be detected in the M1 and M4 cells but not in the V1 and V2 cells (Fig. 1B), implying stabilization of the hPMS2 protein through dimerization with hMLH1 protein. PCR confirmed the presence of exogenous hMLH1 cDNA in the M1 and M4 cells (Fig. 1C), using a forward primer to the vector T7 promoter sequence and a reverse primer to the hMLH1 cDNA exon 1 sequence as described. Additionally, the M1 and M4 cells, as well as the V1 and V2 cells, were comparable with the parental cell line RKO-p with regard to population doubling times (18 ± 2.1 h) and cellular morphology (data not shown).

We next performed clonogenic survival and flow cytometric assays to determine the MMR phenotype after 6-TG treatment in the two hMLH1 transfectants, M1 and M4, compared with vector controls (Fig. 1, D and E). The expression of hMLH1 protein restored typical MMR+ cellular responses after 6-TG treatment for 24 h in both M1 and M4 cells as demonstrated by marked cytotoxicity as assayed by clonogenic sur-
vival (Fig. 1D) and an enhanced G2-M arrest (Fig. 1E) compared with MMR V1 and V2 cells. The M4 cells are more sensitive to 6-TG than are the M1 cells, which may reflect the difference in the levels of hMLH1 protein between the two clones (Fig. 1A). However, both cell lines showed similar MMR responses in other assays performed in this study as described below.

**6-TG Induced a Delayed G2-M Arrest in MMR M4 Cells That Starts on Day 1 and Peaks on Day 3 after 6-TG (3 μM) Removal.** We next examined the time course of the G2-M arrest in response to 3 μM 6-TG in both MMR M4 and MMR V2 cells. As shown in Fig. 2A, in response to 6-TG genotoxicity, MMR M4 cells arrested in G2-M, starting on day 1 (35.6%) and peaking on day 3 (67.6%). The G2-M arrest in the M4 cells was persistent to day 6, without exceeding the day 3 G2-M peak (data not shown). As expected, MMR V2 cells did not demonstrate an increased G2-M arrest in response to 6-TG treatment. Correlated with the prolonged G2-M arrest in the MMR M4 cells, the cell growth curves showed that the cell number in M4 cells did not increase over the 6-day period after 6-TG treatment, whereas the MMR V2 cells kept growing after a 24-h delay, similar to untreated V2 cells (Fig. 2B).

![Fig. 1](image1.png) **Fig. 1** Stable expression of hMLH1 cDNA restores a MMR phenotype in RKO cells. M1 and M4 are hMLH1 cDNA transfectants. V1 and V2 are negative control clones transfected with vector only. HT29 is a hMLH1 wild-type human colon cancer cell line, serving as a positive control. A, Western blot of hMLH1 and hMSH2. Fifty μg of total protein were loaded and separated by 9% SDS-PAGE. B, Western blot of hMLH1 and hPMS2. Actin is a loading control. C, ethidium bromide staining of agarose gel shows PCR product using primers targeting the exogenous hMLH1 cDNA. D, clonogenic survival assays were used to assess changes in the surviving fraction of cells after a 24-h treatment with the indicated doses of 6-TG. The experiments were performed twice, each in triplicate. E, cell cycle distribution at 2 days after 6-TG (3 μM × 24 h) removal. The percentage of cells in G2-M is indicated in each flow cytometry histogram. The data are representative of experiments performed at least three times.

![Fig. 2](image2.png) **Fig. 2** Time course of a G2-M arrest in response to 6-TG compared with IR and VP-16, showing that 6-TG induced a delayed G2-M arrest in MMR M4 cells. A, cell cycle distribution in MMR V2 cells and MMR M4 cells after 6-TG treatment. 6-TG (3 μM) was added 15 h after cell seeding (day −1) and incubated for 24 h. 6-TG was then removed (day 0), cells were harvested daily (days 1–3), and cell cycle profiles were assayed by flow cytometry. Controls were the cells seeded in parallel but without 6-TG treatment. The numbers in the histograms are the proportions of G2-M cells. B, growth curve of V2 and M4 cells after 6-TG treatment. Note that the delay in cell growth in MMR M4 cells treated with 6-TG persists out to day 6, correlating with clonogenic survival data in Fig. 1D. C and D, cell cycle distribution of V2 and M4 cells at early time points after IR (C; 6 Gy) and after VP-16 (D; 5 μM × 2 h). Note that the times of assay by flow cytometry in C and D are in hours, not days as used for 6-TG (A). The data are representative of experiments performed at least three times.
IR is well known to produce DSBs immediately after irradiation, leading to a G2-M arrest (36). We therefore also examined the G2-M arrest in response to IR in the MMR\(^+\) V2 and MMR\(^-\) M4 cells to compare the time course of DSB formation and the G2-M arrest (Fig. 2C). After IR (6 Gy), the G2-M population in both V2 and M4 cells was found to be increased similarly as early as 6 h after IR and peaked 12 h after IR. The time course between IR exposure and the peak G2-M arrest was 12 h, whereas it was 72 h between 6-TG removal and the peak G2-M arrest. Furthermore, we found no significant S-phase delay before a G2-M arrest in M4 cells after 6-TG treatment (Fig. 2A). In contrast, there was a significant S-phase delay after a G2-M arrest after IR exposure in both V2 and M4 cells (Fig. 2C). The correlation of DSB formation and early G2-M arrest was further confirmed using a radiomimetic drug, VP-16, an inhibitor of topoisomerase II. As in the case of IR, VP-16 at 5 \(\mu\)M for 2 h induced a similarly strong early S-phase and G2-M arrest in both MMR\(^+\) V2 and MMR\(^+\) M4 cells within 10 h after treatment (Fig. 2D). These differences imply that there are different signaling pathways for cell cycle regulation in response to 6-TG versus these other DNA-damaging agents.

**Formation of DSBs after 6-TG Treatment Is 6-TG Dose Dependent but not MMR Dependent.** We next carried out PFGE to monitor DSB formation after 6-TG treatment. The DNA sizes measured under our optimized conditions were in the 1–9-Mb range as determined by using DNA size standards in our preliminary experiments (data not shown). As shown in Fig. 3A, immediately after a 24-h exposure to different doses of 6-TG (0–6 \(\mu\)M), DSB formation was detectable in both cell lines and was dose dependent. MMR\(^+\) V2 cells show slightly greater DSB formation than MMR\(^+\) M4 cells under the conditions used. The time course of DSB formation after 3 \(\mu\)M 6-TG treatment revealed that DSBs decreased in both cell lines on day 1 and day 2 after 6-TG removal (Fig. 3, B and C). Cell viability was monitored throughout the 3-day period using a trypan blue exclusion assay, showing about 5% cell death in both cell lines.

Because DSBs could effectively induce a G2-M arrest in the M4 cells within 12 h after 6 Gy IR (Fig. 2B) but not after 3 \(\mu\)M 6-TG treatment (Fig. 2A), we questioned whether it was because IR induced more DSBs than 6-TG did. We therefore compared DSB formation in the M4 cells immediately after treatment with either IR or 6-TG. The results in Fig. 3D show that 6 Gy of IR actually caused fewer DSBs than 3 \(\mu\)M 6-TG \(\times\) 24 h did in the M4 cells. These results further suggest that DSB formation measured early after 6-TG treatment is not associated with the G2-M arrest occurring at later times. Interestingly, we repeatedly observed that the DSB formation immediately after 6-TG treatment (day 0) was slightly greater in MMR\(^-\) V2 cells than in MMR\(^+\) M4 cells. The reason for this is not clear. The bar graph shown in Fig. 3C is generated from a representative gel (Fig. 3B). We did not attempt to average the PFGE data from individual experiments because there are several interexperiment variables (e.g., proteinase digestion, ethidium bromide staining/destaining, and scanning technique) that limit data collection. However, in each individual experiment (gel), we observed the same trend.

A comparison of the 6-TG dose response (Fig. 3A) and time course (Fig. 3B) of DNA fragmentation by PFGE in our MMR\(^+\) M4 cells and MMR\(^-\) V2 cells suggests that 6-TG-related DSB formation is independent of MMR and MMR-mediated cytotoxicity (Fig. 1D). Furthermore, DSB formation was greater on day 0 in both cell lines immediately after 6-TG treatment (Fig. 3, B and C), whereas a 6-TG-induced G2-M arrest started on day 1 and peaked on day 3 after 6-TG removal in the M4 cells (Fig. 2A). The time difference between these two events (DSB formation and the peak G2-M arrest) was as long as 72 h, suggesting that 6-TG-related DSB formation is also not temporally related to the observed G2-M arrest in the MMR\(^+\) M4 cells in response to 6-TG treatment. The DSB formation observed early after 6-TG treatment in both MMR\(^+\) M4 and MMR\(^-\) V2 cells may be from 6-TG-induced purine deprivation (37).

**MMR\(^+\) Cell Lines Demonstrate Robust and Persistent SSBs after 6-TG Treatment.** We next used the alkaline Comet assay to detect both DSBs (as two SSBs) and SSBs after 6-TG treatment protocols similar to those used for PFGE. Because the M4 cells showed a level of DSB formation similar to that of V2 cells by PFGE after 6-TG treatment (Fig. 3, B and C),
any additional strand breaks detected by the alkaline Comet assay in the M4 cells should represent the formation of DNA SSBs. Although the alkaline Comet assay can also detect alkali-labile sites, these lesions, if any, should be similar in both cell lines because they are not MMR dependent. In each experiment, the V2 and M4 cells mixed with agarose were placed on the same slide and then electrophoresed and handled under identical conditions. As shown in Fig. 4A, both MMR+ and MMR− cell lines exhibited global but small DNA tails on day 0, consistent with the PFGE result that showed comparable DSB formation in both cell lines on day 0 (Fig. 3B). However, the DNA tails increased significantly after 6-TG treatment in MMR+ M4 cells but regressed in MMR− V2 cells. Thus, as the number of DSBs decreased in the 2 days after removal of 6-TG (3 μM × 24 h) in both MMR+ and MMR− cells by PFGE (Fig. 3A), the extent of DNA migration by alkaline Comet assay (quantitated as tail moment) increased in MMR+ M4 cells (Fig. 4B), suggesting different kinetics of DNA SSB formation compared with DNA DSB formation in these MMR+ cells after 6-TG treatment. In addition, the time course of SSB formation was closely correlated with the time course of the 6-TG-induced G2-M arrest in the M4 cells (Fig. 2A).

DISCUSSION
In this study, we attempted to determine the molecular mechanism underlying the MMR-mediated, 6-TG-induced G2-M arrest, i.e., what signal is introduced by functional MMR upon recognition of drug-induced DNA lesions to trigger a G2-M arrest. Using PFGE and alkaline Comet assays, we examined the extent and time course of DSBs and SSBs induced in the MMR+ and MMR− cells after 6-TG treatment. We found that DSB formation (PFGE assay) occurred to a similar extent and time course in our MMR+ M4 and MMR− V2 cells, i.e., DSB formation was greater immediately after 6-TG treatment and decreased in the 2 days after 6-TG removal (Fig. 3). We also found that total DNA strand breaks (as determined by the alkaline Comet assay) were similar in the MMR+ M4 and MMR− V2 cells immediately after 6-TG treatment, consistent with our PFGE assay results (Fig. 4). However, in the subsequent 2 days after 6-TG removal, the Comet tail moment increased significantly in MMR+ M4 cells, whereas it regressed in MMR− V2 cells (Fig. 4B). Although only data on the V2 and M4 cell lines are shown here, MMR− V1 and MMR+ M1 cell lines showed similar differences. Taken together, these data suggest that more SSBs were produced in MMR+ cells at later times after 6-TG exposure. The time course of SSB generation was temporally correlated with the time course of 6-TG-induced G2-M arrest, which increased later (not earlier) after 6-TG removal in the MMR+ M4 cells (Fig. 2A), suggesting that MMR mediates a 6-TG-induced G2-M arrest by introducing DNA SSBs upon recognition of the drug-induced mismatches. In this context, SSBs can be considered a secondary signal produced by MMR after recognition of the primary signals, i.e., the 6-meTG mismatches. Thus, the formation of DNA SSBs may provide a link between initial MMR recognition of 6-TG-induced mismatches and the subsequent G2-M arrest signaling. It is unlikely that the persistent SSB demonstrated in MMR+ M4 cells is a normal repair intermediate because MMR has been shown in vitro to be able to reach maximal repair efficiency of roughly 50% within 15 min (38).

In our experiments, SSB formation in MMR+ cells was estimated by comparison of total strand breaks (measured by alkaline Comet assay) with DSBs (measured by PFGE) in MMR+ versus MMR− cells. Previous reports (39–41) using the alkaline elution assay for SSB formation lend supportive data to our conclusions in MMR+ cells, although these early experiments did not involve comparative studies between genetically matched MMR+ and MMR− human tumor cells. For example, Christie et al. (39) treated Chinese hamster ovary cells with 6-TG for 24 h and then measured SSB at 12 h after 6-TG removal, revealing 6-TG dose-dependent SSB formation. Additionally, Fairchild et al. (40) reported increasing SSB production in L1210 cells treated with increasing doses of 6-TG for 12 h and then maintained in drug-free medium for 12 h. Finally, Pan and Nelson (41) characterized DNA damage in 6-TG-treated cell lines including Chinese hamster ovary cells, L1210 cells, and two human lymphoblastoid cell lines, HL60 and CEM. Their data indicated that SSBs, not DSBs, were formed later (24 h), not earlier (4 h), after 6-TG treatment in the daughter DNA. Because the cell lines used in these studies showed a marked cytotoxic response to 6-TG, we assume that these cell lines are MMR+ clones. Therefore, the previous data are consistent with our data in the MMR+ clones.

The present report is the first comparative study between MMR+ and MMR− isogenic human tumor cells to investigate
the MMR-mediated, drug-induced G2-M arrest signal. Our results support the model of futile cycle repair for MMR recognition of chemically induced mismatches. The possible sequential events could be as follows. 6-TG is incorporated into a daughter DNA strand in the first cell cycle and subsequently methylated to 6-meTG. In the following DNA replication cycle, 6-meTG on the parental strand is matched with a normal base T or C to form 6-meTG mismatches. MMR recognizes the mismatches and carries out the repair process (incision, excision, synthesis, and ligation) on the daughter strand. Because 6-meTG in the parental strand is not removed, MMR repeatedly processes this damage at the same site, leading to confined SSB formation in the daughter strand to activate a G2-M checkpoint signaling cascade. However, our data do not rule out the possibility that the assembly of a repair protein complex per se (as proposed in the alternative MMR model) may be a signal after other drug or DNA-damaging (e.g., IR) agents, as we and others have published previously (14–17, 20, 33, 35).

In this study, we also established hMLH1+ RKO cell lines through transfection of hMLH1 cDNA into hMLH1− RKO cells. To our knowledge, this is the first report of a hMLH1 cDNA-corrected, hMLH1+ RKO cell model. To ensure that the cell model established is truly isogenic, we subcloned the parental RKO cell line and chose one clone to start the transfection. As an experimental note, we have attempted to transfec the same pcDNA3.1/hMLH1 plasmid into similarly isolated subclones of hMLH1+ HCT116 and A2780/CP70 cell lines. We failed to obtain stably expressing clones in HCT116 cells, although we could detect strong but transient hMLH1 protein expression in this cell line. This result is similar to that reported by Zhang et al. (42), where hMLH1 cDNA-transfected HCT116 cells demonstrated enhanced apoptosis. In A2780/CP70 cells, we selected three stable hMLH1-expressing clones; however, none of them exhibited a MMR phenotype after 6-TG treatment. This result is in agreement with a report from Branch et al. (35), where hMLH1 cDNA expression did not sensitize A2780/CP70 cells to cisplatin. Because both HCT116 and A2780/CP70 cells have been made MMR− through a transfer of an entire human chromosome 3, it is possible that there was loss of one or more unidentified protein partners that assist with MMR function and have genes located on the chromosome 3.

In summary, we established MMR+ RKO cell lines through transfection of hMLH1 cDNA. We defined the MMR− phenotype in the transfected M1 and M4 cells as showing greater cytotoxicity (up to 1 log cell kill) and a prolonged G2-M cell cycle arrest for up to 6 days after 6-TG treatment compared with MMR− vector controls. We performed PFGE and alkaline Comet assays to examine the extent and time course of DNA strand breaks after 6-TG treatment. We found comparable levels of DNA DSBs immediately after treatment with 3 μM 6-TG in MMR− versus MMR+ (minus) cells, which then decreased over the next 2 days. However, DNA SSB formation was more frequent and longer lived in MMR+ cells compared with MMR− cells. The time course of SSB formation closely correlated with the time course of G2-M arrest in MMR+ M4 cells, suggesting a causal relationship between the two events. Our results suggest that MMR mediates 6-TG cytotoxicity by introducing SSB at the site of 6-TG mismatches and subsequently activates signaling for a G2-M arrest and enhanced cell death.

REFERENCES

DNA Mismatch Repair (MMR) Mediates 6-Thioguanine Genotoxicity by Introducing Single-strand Breaks to Signal a G_2-M Arrest in MMR-proficient RKO Cells

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