Evidence for a Connection between the Mismatch Repair System and the G₂ Cell Cycle Checkpoint¹

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Abstract

The human colon tumor cell line HCT116 is deficient in wild-type hMLH1, is defective in mismatch repair (MMR), exhibits microsatellite instability, and is tolerant to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Transferring a normal copy of hMLH1 on chromosome 3 into the cell line restores MMR activity, stabilizes microsatellite loci, and increases the sensitivity of the cell to MNNG. Previous studies in other cell lines tolerant to alkalyating agents such as MNNG or N-methyl-nitrosourea have shown cross-tolerance to 6-thioguanine (6TG), leading to a hypothesis that sensitivity to MNNG or 6TG may be the result of MMR deficiency. To test this hypothesis, we studied the effects of 6TG on the MMR-tolerant, MMR-deficient HCT116 cell line and its MMR-sensitive, MMR-proficient, MNNG-tolerant, and MMR-deficient derivatives. Continuous exposure to low doses of 6TG (0.31–1.25 μg/ml) had no apparent effect on colony-forming ability (CFA) in MNNG-tolerant, MMR-deficient cells, whereas MNNG-sensitive, MMR-proficient cells exhibited a dose-dependent decrease in CFA. Growth kinetics and cell cycle analysis revealed that the growth of 6TG-treated HCT116+chr3 cells was arrested at G2 after exposure to low dose of 6TG. In contrast, the same exposure to 6TG did not induce G2 arrest but rather a G1 delay in HCT116 and HCT116+chr2. To obtain further evidence for the role of MMR on 6TG and MNNG toxicity, we isolated an MNNG-resistant revertant clone, M2, from the MNNG-sensitive, MMR-proficient HCT116+chr3 cell line and characterized the MMR activity, hMLH1 status, and 6TG response. The results showed that M2 cells lost MMR activity as well as the previously introduced normal hMLH1 gene. Restoration of the CFA of M2 and an absence of G2 arrest were observed after treatment with low doses of 6TG.

The results further suggest that any agent that induces DNA mispairs will cause G2 arrest in MMR-proficient cells but not in MMR-deficient cells.

Introduction

HNPCC¹ tumors exhibit a high rate of mutation at microsatellite sequences (1–3). This phenotype has been linked to an inherited germline mutation in one of the MMR genes, hMLH1, hMSH2, hPMS1, or hPMS2, in which a subsequent somatic mutation in the wild-type allele occurs before tumor formation (4–6). Microsatellite instability has been reported to occur in approximately 10–15% of sporadic colon neoplasias (2, 3), and has also been linked to a defect in mismatch repair in sporadic colon and endometrial tumor cell lines (7–9). Previous work with the human colon cancer cell line HCT116, which has no normal hMLH1 gene product, has shown that this line is deficient in MMR (8, 9), exhibits microsatellite instability, and is tolerant to the methylating agent MNNG (7). These studies suggested that inactivation of both copies of the hMLH1 gene leads to complete loss of mismatch repair activity, and that such a defect is responsible for the microsatellite instability found in HNPCC and some sporadic tumors. Consistent with this hypothesis, we previously demonstrated that the introduction of one copy of the wild-type hMLH1 gene on chromosome 3 into HCT116 cells (HCT116+chr3) restored mismatch repair activity and lowered mutation frequency at a microsatellite locus (7). Tolerance to alkalyating agents such as MNNG has been observed in cell lines (10, 11) that have also been shown to be deficient in MMR and demonstrate microsatellite instability (9, 12, 13). Previously, we demonstrated that transfer of chromosome 3 into HCT116 cells diminished the tolerance of the cells to MNNG toxicity. After MNNG treatment, these cells exhibit prolonged growth arrest at G2, followed by eventual cell death (7), suggesting that the mismatch repair system is involved in mediating MNNG toxicity.

Alkalyating agent-resistant cells have been shown to be cross-tolerant to the guanine base analogue 6TG (11, 14, 15). O'-methylguanine, the major methylation product from MNNG adduct formation (12), has a similar molecular volume to 6TG (16). Both 6TG and O2'-methylguanine are unable to form stable base pairs with either of the pyrimidines (16). Several investigators have hypothesized that tolerance to 6TG and O2'-methylguanine may be the result of a defect in the MMR system (11, 13, 15). To test this hypothesis, we examined whether MMR-deficient, MNNG-tolerant HCT116 cells were also tolerant to low doses of 6TG and whether MMR-proficient, MNNG-sensitive HCT116 containing the transferred chromosome 3 (HCT116+chr3-6) were sensitive to the same doses of 6TG. In addition, we isolated and characterized an MNNG-resistant revertant clone from HCT116+chr3-6 cells. Using these HCT116 cell line derivatives, we then addressed whether the MMR system mediates 6TG toxicity by examining the colony-forming ability and cell cycle progression after treatment with 6TG.

Materials and Methods

Chemical Reagents. 6TG and 8AG (Sigma Chemical Co., St. Louis, MO) were dissolved in 0.1 N NaOH and stored at −10°C. MNNG (Aldrich Chemical Co., Milwaukee, WI) was dissolved in DMSO (Sigma) and stored at −10°C. Materials and Methods

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3. The abbreviations used are: HNPCC, hereditary nonpolyposis colon cancer; MMR, mismatch repair; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; 6TG, 6-thioguanine; 8AG, 8-azaguanine; IMDM, Iscove’s modified Dulbecco’s medium; FBS, fetal bovine serum; Aprt, hypoxanthine phosphoribosyl transferase; HAT, hypoxanthine aminopterin thymidinase; SSCP, single-strand conformational polymorphism.
normal human chromosome as described previously (7). These cell cultures were maintained in IMDM containing 10% FBS and G418 (400 μg/ml; Gibco-BRL). The MNNG-resistant revertant cell line derived from HCT116+chr3-6, HCT116+chr3-6-M2, was maintained in IMDM containing 10% FBS and G418. HAT medium (Sigma) was dissolved in IMDM containing 10% FBS at a final concentration of 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine.

6TG Cytotoxicity Assay. Exponentially growing cell lines were plated in duplicate at densities of 10^2 to 10^5 on 6-cm plates and 10^4 to 10^6 on 10-cm plates. After allowing for attachment to the plate for 18–24 h, the medium was replaced with fresh medium containing 6TG (0.31–10 μg/ml). Cells were maintained in 6TG-containing medium for 10 days, changing the medium every 3 days. The plates were washed with PBS, fixed with methanol, and stained with 3% Giemsa. Colonies with greater than 50 growing cells were counted and expressed as a ratio of the plating efficiency for untreated cells.

Selection of 6TG-resistant Clones. Cells lines were plated (10^2 to 10^5) on 15-cm plates and treated with 6TG (1.25 μg/ml) for 24 and 48 h. The cells were trypsinized and treated with protease K (Gibco-BRL) at 55°C overnight. DNA was extracted with phenol-chloroform, precipitated with ethanol, dried, and resuspended in TE (10 mM Tris-Cl, 1 mM EDTA) buffer. DNA was quantitated using a diphenylamine (Sigma) assay, which is highly specific for DNA (17). Briefly, equal volumes of sample DNA solutions, including a standard human Cot 1 DNA (Gibco-BRL; 0–20 μg/ml) and 10% perchloric acid were incubated at 70°C for 30 min. Two volumes of a diphenylamine/acetaldehyde solution were added. Samples were incubated in the dark overnight, and the absorbance at 600 nm was measured by spectrophotometry. The concentration of standard DNA was calculated from the standard curve constructed from known concentrations of Cot 1 DNA. DNA from 6TG-treated cells and known amount of 6TG (0.1–10 μg/ml) were dissolved in 0.1 N HCl and heated to 70°C for 30 min. The absorbance at 346 nm (the peak absorbance for 6TG; Ref. 14) was measured, and the amount of 6TG in cellular DNA was determined using the standard curve constructed from the known amount of 6TG.

Results and Discussion

Correlation between MMR Activity and 6TG Toxicity. To test whether MNNG sensitivity and MMR activity are correlated with 6TG sensitivity, we first compared colony-forming ability in the presence of low concentrations of 6TG (0.31–1.25 μg/ml) among MNNG-resistant, MMR^− cells (HCT116 and HCT116+chr2) and MNNG-sensitive, MMR^+ cells (HCT116+chr3). As shown in Fig. 1A, HCT116 and two independent clones from HCT116+chr2 showed no significant decrease in colony-forming ability after continuous exposure to low doses of 6TG. In contrast, two clones from HCT116+chr3 showed a dose-dependent decrease in colony-forming efficiency. Ten growing subclones from HCT116 and HCT116+chr2 were isolated after exposure to 1.25 μg/ml of 6TG and cultivated in HAT medium, which is selectively cytotoxic to hprt-deficient cells. All clones were resistant to HAT selection, indicating that tolerance to 6TG was not due to inactivation of hprt gene.

Another guanine analogue, 8AG, which is also phosphoribosylated by hprt and selected for hprt-deficient cells, is known to exert its primary toxicity by purine starvation (20). Treatment with 8AG at 1–5 μg/ml had similar effects on colony-forming ability in MMR-proficient and MMR-deficient cells (Fig. 1B). These results indicate that MMR deficiency specifically correlates with 6TG tolerance but not 8AG toxicity.

To determine whether tolerance to 6TG in MMR^− cells was conferred by differential 6TG incorporation into DNA, the content of 6TG in high molecular weight DNA from 6TG-treated cells was measured and compared. HCT116 and HCT116+chr3 had similar incorporation of 6TG after 24 and 48 h of exposure (Fig. 2). hprt-deficient clones derived from HCT116 served as negative control and had no appreciable incorporation of 6TG under the same conditions of culture.

6TG is known to exert its toxicity by two different mechanisms. 6TG, as well as 8AG, can cause purine starvation in the nucleic acid base pool and directly leads to cell death (19). A second mechanism is thought to involve incorporation of 6TG into cellular DNA, but this process is not well understood (20). Because incorporated 6TG is thought to mimic DNA mispairs (16) and 8AG is not readily incorporated into DNA as 6TG (21), our results suggest that MMR is involved in mediating the second mechanism for toxicity in which the

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Fig. 1. A, percent colony-forming ability after 10 days of continuous exposure to 6TG (0.31, 0.625, and 1.25 μg/ml) is depicted in MMR-deficient cell lines (HCT116, HCT116+chr2-1, HCT116+chr3-3, and M2) and MMR-proficient cell lines (HCT116+chr3-5 and HCT116+chr3-6). HCT116+chr3, clones 5 and 6, demonstrates significantly decreased colony-forming ability when compared to HCT116 MMR-deficient cells at 0.625 and 1.25 μg/ml of 6TG (*, P < 0.01; **, P < 0.005). B, percent colony-forming ability after 10 days of continuous exposure to SAG (0.31-5 μg/ml) in HCT116, HCT116+chr2-1, and HCT116+chr3-6. No statistically significant difference was observed among the groups for concentrations up to 2.5 μg/ml. The difference seen at 5 μg/ml is not significant but most likely represents the difference in mutation rate at the hprt locus.

Fig. 2. A representative 6TG incorporation experiment is shown. HCT116, HCT116+chr3-6, and M2 all have similar incorporation of 6TG into DNA after 24 and 48 h of 6TG exposure. hprt-deficient cells are unable to use 6TG in DNA synthesis. An hprt-deficient clone derived from HCT116 (HCT116 hprt') was assayed as a negative control and has no appreciable incorporation of 6TG after 24 and 48 h of exposure.

Fig. 3. Repair efficiencies in extracts of the HCT116 cell lines are shown above. Mock, negative control; HeLa cell extract, positive control. As illustrated, HCT116 is repair deficient, HCT116+chr3 (clone 6) is repair proficient, and M2 is repair deficient. HEC59 cell extract, which is known to be hMSH2 deficient, complements M2 cell extract, indicating that the loss of repair for M2 is not due to hMSH2 inactivation.

induction of mispairs and their recognition by the MMR system is crucial.

MNNG Resistance Correlates with 6TG Tolerance, Loss of MMR Activity, and Wild-type hMLH1. To obtain further evidence for the role of MMR in MNNG and 6TG toxicity, we isolated MNNG-resistant clones from MNNG-sensitive, MMR+ HCT116+chr3 cells and then examined whether MNNG resistance correlated with loss of MMR activity and the reacquisition of 6TG tolerance. HCT116+chr3, clone 6 cells were treated with 5 μM MNNG for 45 min at 37°C. Most of the treated cells showed growth arrest and gradual cell death. Two weeks after treatment, growing colonies were obtained at a frequency of about 10^-4 treated cells. Ten such clones were independently isolated and subjected to MNNG treatment (5 μM 45 min at 37°C). Eight of these clones showed no growth arrest after MNNG treatment.

One such clone, M2, was chosen for further characterization. Colony-forming ability in MNNG and 6TG was determined. M2 exhibited tolerance to both 6TG (Fig. 1A) and MNNG (not shown) that was similar to HCT116 cells. M2 had similar 6TG incorporation into its DNA, as did the parental HCT116+chr3 cells (Fig. 2), eliminating the possibility that tolerance was due to a lack of 6TG incorporation.

We next compared MMR activity in extracts from M2 cells to that of extracts of control cells. A circular M13mp2 DNA substrate was used, containing a covalently closed (+) strand and a (-) strand with a nick (to direct repair to this strand) located several hundred base pairs away from the G-G mispair at position 88 in the lacZ α-complementation coding sequence. The (+) strand encodes a colorless plaque phenotype, whereas the (-) strand encodes a blue plaque phenotype. If the un repaired heteroduplex is introduced into an E. coli strain deficient in methyl-directed heteroduplex repair, plaques will have a mixed plaque phenotype on selective plates due to expression of both strands of the heteroduplex. However, repair occurring during incubation of the substrate in a repair-proficient human cell extract will reduce the percentage of mixed plaques and increase the ratio of the (+) strand phenotype (colorless with this substrate) relative to that of the (-) strand phenotype (blue) because the nick directs repair to the (-) strand.

Mismatch repair is readily detected in a HeLa cell extract (22), as indicated by the reduction in mixed plaque phenotypes and the change in the blue:white plaques ratio when compared to an un repaired control heteroduplex (Fig. 3). Most important, an extract of M2 cells is defective in mismatch repair, as indicated by both a high percentage
of mixed plaque phenotypes and little change in the blue:white plaques ratio (Fig. 3). The repair deficiency in M2 cell extracts was complemented in reactions, to which were added an equal amount of a second (HEC59 cell) extract that lacks repair activity and has a known defect in the hMSH2 gene and protein (9). This result indicates that the M2 defect is not in the hMSH2 gene.

To determine whether M2 lost the previously introduced normal copy of hMLH1, PCR products from DNA segments encompassing codons 228–263 of hMLH1 from HCT116, HCT116+chr3, and M2 were analyzed using SSCP analysis. As shown in Fig. 4, HCT116 exhibits one band on SSCP analysis, HCT116+chr3 exhibits two bands, the lower one representing the wild-type hMLH1, and M2 lost the wild-type hMLH1 segment present in HCT116+chr3. All of the above data demonstrate complete correlation between the presence of the normal hMLH1 gene, proficiency of MMR activity, and sensitivity to MNNG and 6TG in HCT116 and its derivatives. These results strongly support the hypothesis that the MMR system mediates both 6TG and MNNG toxicity.

6-TG Induces G2 Growth Arrest in MMR-proficient Cells. We next examined the growth kinetics of MMR+ and MMR− HCT116 cells in response to continuous exposure to low dose 6TG (0.625 µg/ml). As shown in Fig. 5A, all of the cell lines tested were growth inhibited compared to untreated cells. However, MMR+ HCT116+chr3 cells underwent a morphological change characterized by enlarging and flattening of the cells and accumulation of cytoplasmic vacuoles. HCT116+chr3 did not double their cell number, even after 4 days of treatment, whereas HCT116, HCT116+chr2, and M2 cells doubled their population after 1–2 days of treatment.

Preliminary findings reported in our previous study revealed that MNNG-treatment also induced a flat cellular morphology in HCT116+chr3 cells, and this corresponded to G2 arrest (7). To determine whether 6TG induces G2 arrest in HCT116+chr3 cells, we performed cell cycle analysis on MMR+ and MMR− HCT116 cell lines after continuous exposure to 0.625 µg/ml of 6TG. As shown in Fig. 5B, 32% of HCT116+chr3 cells arrested at G2 by 48 h and 73% by 96 h, whereas 20% of the untreated cells were in G2. Because 6TG-treated HCT116+chr3 cells did not double their population by 96 h, these cells might arrest at the first G2 after 6TG incorporation. In contrast, none of the MMR− cell lines arrested in G2, but all showed a G2 arrest at both 48 and 96 h after treatment (Fig. 5B).

These results concur with previous studies that 6TG has a dual...
effect on cell cycle kinetics (20, 23). Our results demonstrate that the G2 effect is MMR dependent, and the G1 arrest is independent of the MMR system. The absence of the G2 effect in HCT116+chr3-6 cells after 6TG treatment may be due to the fact that these cells are arrested at the first G2 after 6TG incorporation.

Recently, it has been shown that hMSH2 specifically binds to mismatches (24). Since O6-methylguanine, a methylation product of MNNG, and 6TG are known to form unstable base pairs with other purines or pyrimidines when incorporated into cellular DNA, it may be that hMSH2 binds to environmentally induced mismatches and mediates its repair function in collaboration with the other MMR proteins, including hMLH1 (25). Our results suggest that MMR not only repairs DNA damage but also recognizes DNA damage and is involved in a process that signals G2 arrest. The MMR protein complex might interact directly or indirectly with other proteins or protein complexes that are responsible for the G2 checkpoint. Alternatively, G2 arrest may be signaled by DNA strand breaks generated by mismatch repair activity (26). Further studies are needed to identify the cellular mechanisms that result in G2 arrest in response to the presence of inappropriate base pairing in DNA.

We used HCT116+chr3 cells containing one copy of wild-type hMLH1 as MMR+ cells. The heterozygous state of the hMLH1 locus in HCT116+chr3 cells is similar to that of the cells from HNPCC patients. As shown in this study, a single treatment with MNNG selected MMR+ cells (i.e., M2 cells) from MMR+ HCT116+chr3 cells. M2 lost the normal hMLH1 that had been introduced. It is not clear whether MNNG treatment is the cause of hMLH1 loss; however, these results indicate that MNNG allowed selective clonal expansion of the subpopulation of MMR+ cells present in HCT116+chr3. This implies that agents that induce DNA damage that mimics mismatches may present a risk to cells that are heterozygous for MMR gene loci, such as all cells in HNPCC patients. These agents might not only induce mutations in the wild-type allele, but may simultaneously select for clonal expansion of those cells which incurred inactivation of that allele.

In summary, these data provide evidence for a connection between the MMR system and the G2 checkpoint. Our results also suggest that agents that cause mismatches in cellular DNA may induce G2 growth arrest in MMR-proficient but not in MMR-deficient cells. The link between the MMR system and G2 arrest suggests that the MMR system is critical not only for DNA repair, but has additional functions that limit the replication of cells with drug-induced mismatches that can not be repaired.

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