Loss of DNA Mismatch Repair Imparts Defective cdc2 Signaling and G2 Arrest Responses without Altering Survival after Ionizing Radiation

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ABSTRACT

Our previous data demonstrated that cells deficient in MutL homologue-1 (MLH1) expression had a reduced and shorter G2 arrest after high-dose-rate ionizing radiation (IR), suggesting that the mismatch repair (MMR) system mediates this cell cycle checkpoint. We confirmed this observation using two additional isogenetically matched human MLH1 (hMLH1)-deficient and -proficient human tumor cell systems: human ovarian cancer cells, A2780/CP70, with or without ectopically expressed hMLH1, and human colorectal carcinoma cells, RKO, with or without azacytidine treatment to reexpress hMLH1. We also examined matched MutS homologue-2 (hMSH2)-deficient and -proficient human endometrial carcinoma HEC59 cell lines to determine whether hMSH2, and MMR in general, is involved in IR-related G2 arrest responses. As in MLH1-deficient cells, cells lacking hMSH2 demonstrated a similarly altered G2 arrest in response to IR (6 Gy). These differences in IR-induced G2 arrest between MMR-proficient and -deficient cells were found regardless of whether synchronized cells were irradiated in G0/G1 or S phase, indicating that MMR indeed dramatically affects the G2-M checkpoint arrest. However, unlike the MMR-dependent damage tolerance response to 6-thioguanine exposures, no significant difference in the clonogenic survival of MMR-proficient cells compared with MMR-proficient cells was noted after high-dose-rate IR. In an attempt to define the signal transduction mechanisms responsible for MMR-mediated G2 arrest, we examined the levels of tyrosine 15 phosphorylation of cdc2 (phospho-Tyr15-cdc2), a key regulator of the G2-M transition. Increased phospho-Tyr15-cdc2 levels were observed in both MMR-proficient and -deficient cell lines after IR. However, the levels of phospho-Tyr15-cdc2 rapidly decreased in MMR (hMLH1 or hMSH2)-deficient cell lines at times coincident with progress from the IR-induced G2 arrest through M phase. Thus, differences in the levels of phospho-Tyr15-cdc2 after high-dose-rate IR correspond temporally with the observed differences in the IR-induced G2 arrest, suggesting that MMR proteins may exert their effect on IR-induced G2 arrest by signaling the cdc2 pathway. Although MMR status does not significantly affect the survival of cells after high-dose-rate IR, it seems to regulate the G2-M checkpoint and might affect overall mutation rates.

INTRODUCTION

MMR is a highly conserved repair system that corrects mismatches arising during DNA replication and safeguards genomic integrity (1–4). Defective MMR is strongly associated with HNPCC and is also found in many types of sporadic human cancers caused by hypermethylation silencing (5–10). MMR consists of at least seven proteins, including hMLH1, hMLH3, hPMS1, hPMS2, hMSH2, hMSH3, and hMSH6. hMLH1 and hMSH2 are the two major HNPCC susceptibility genes. Of ~300 mutations in HNPCC described to date, ~50% involve the hMLH1 gene; ~40% involve the hMSH2 gene, and the remainder are mostly found in hMLH6 and hPMS2 (11).

MMR proteins function as heterodimeric complexes. hMLH1 can form a heterodimer with hPMS2 (hMutLβ), hPMS1 (hMutLβ), or hMLH3 (12). The three types of hMutL complexes are, presumably, functionally redundant. hMLH1 is, therefore, an essential component of the hMutL protein complex. hMSH2 can form a heterodimer with either hMSH6 (hMutSα) or hMSH3 (hMutSβ; Ref. 13). hMutSα primarily recognizes base/base mismatches, whereas hMutSβ primarily recognizes large insertion/deletion loops. Thus, hMutS plays a central role in initiating mismatch recognition and binding, and hMutL acts as a molecular matchmaker between hMutS and downstream proteins to complete the repair process (1, 4). Both hMSH2 and hMLH1 are, therefore, critically important to MMR activity as reflected by their predominant alteration in HNPCC patients, as well as in most sporadic tumors with microsatellite instability.

Studies over the last decade have suggested that MMR function is also implicated in other cellular processes, such as DNA recombination (14), meiosis (15), transcription-coupled repair (16), and damage responses to chemical and physical agents (17, 18). We reported previously that hMLH1-deficient cells showed a reduced and shorter G2 arrest after IR compared with hMLH1-proficient cells, suggesting a possible role for MMR (hMLH1) in mediating a G2-M cell cycle checkpoint arrest (18). The G2-M cell cycle checkpoint arrest in response to IR can involve several DNA damage-response pathways (19, 20). The ATM/ATR-cdc2 signaling pathway (21, 22), p53/p21 (23, 24), GADD45 (25), hRAD9 (26), mitogen-activated protein kinase (27), BRCA1 (28), and RINT-1 (29) have all been shown to affect an IR-induced G2-M arrest. In addition, our previous data suggested that one of the MMR proteins, hMLH1, also played a role in the regulation of a proficient IR-induced G2-M arrest (18). However, several questions remained regarding the interaction of MMR and an IR-induced G2-M arrest. First, was the IR-induced G2-M arrest a general property of MMR, or a novel function of the hMLH1 protein? Second, was the observed differential G2-M arrest after IR between MMR-proficient and -deficient cells associated with differences in a G1 arrest or an S-phase delay? Third, by what signal transduction pathway did MMR initiate G2-M arrest responses? In the present study, we show that hMSH2-deficient human tumor cells demonstrate a reduced and shorter G2-M arrest in response to high-dose-rate IR, similar to hMLH1-deficient human tumor cells. These data strongly suggest that MMR mediates a G2-M arrest in cells after high-dose-rate IR. We also show that the differences in IR-induced G2-M arrest between MMR-proficient and -deficient cells occurred regardless of whether synchronized cells were irradiated in G0/G1 or S-phase, indicating that MMR indeed has an impact on the G2-M checkpoint arrest. Furthermore, we provide the first evidence that the kinetics of tyrosine 15 phosphorylation of the cdc2 protein, known to control mitotic entry from G2, differ after IR between MMR-proficient and -deficient cells. This observation suggests that MMR mediates an IR-induced G2 arrest by interacting with the cdc2 signaling pathway.
**Materials and Methods**

**Cell Lines.** A cisplatin-resistant human ovarian cancer cell line, (A2780/CP70, hMLH1-deficient caused by hypermethylation of the gene promoter), its hMLH1-proficient counterpart (CP70/CH3), carrying a transferred human chromosome 3 which contains the hMLH1 gene, and its negative control (CP70/CH2, hMLH1-deficient, carrying a transferred human chromosome 2) were kindly gifts of Dr. Robert Brown (Glasgow University, Glasgow, United Kingdom). These cell lines were derived and characterized for MMR-dependent responses to cisplatin (30), and will be referred to as CP70, CH3, and CH2, respectively. RKO, a human colorectal carcinoma cell line, deficient in hMLH1 because of hypermethylation of the gene promoter region (31), was also examined. A RKO hMLH1-proficient counterpart (RKO/aza50) was isolated after two consecutive 2-day treatments with 0.1 \( \mu \text{g/ml} \) 5-deoxyazacytidine in our laboratories. RKO/aza50 was carried for 9 months without additional azacytidine treatment and stably expressed hMLH1 protein, as well as retained sensitivity to 6-TG. The human endometrial carcinoma HEC59 cell line (hMSH2-deficient because of mutations in both alleles of the gene) and its hMSH2-proficient counterpart HEC59/2-4 (carrying a transferred human chromosome 2 that contains the hMSH2 gene) were established and generously provided by Dr. Thomas Kunkel (National Institute on Environmental Health Sciences, Research Triangle Park, NC; Ref. 32).

CP70, CH2, and CH3 cell lines were grown in RPMI 1640 supplemented with 10% defined FBS (HyClone, Logan, UT), 1 mM glutamine, and 1 mM nonessential amino acids in 5% CO\(_2\) at 37°C. CH2 and CH3 cell lines were maintained under hygromycin (250 \( \mu \text{g/ml} \)) selection. All other cell lines were grown in DMEM supplemented with 10% FBS, 1 mM glutamine, 1 mM nonessential amino acids in 10% CO\(_2\) at 37°C. The HEC59/2-4 cell line was maintained under G418 (250 \( \mu \text{g/ml} \)) selection. The population doubling times for these cell lines were as follows: (a) the CP70 cell system, 13.5 \pm 1.6 h; (b) the RKO cell system, 18 \pm 2.1 h; and (c) the HEC59 system, 28.7 \pm 2.6 h.

**Antibodies.** The antibodies used in this study were as follows: (a) anti-hMLH1 and anti-hPMS2 (BD PharMingen, San Diego, CA); (b) anti-hMSH2 (Calbiochem, San Diego, CA); (c) anti-hMSH6 (BD Transduction Laboratories, Lexington, KY); (d) anti-tubulin and anti-actin (Sigma Chemical Co., St. Louis, MO); (e) anti-phospho-Tyr15-cdc2 (Cell Signaling Technology, Beverly, MA); and (f) anti-cdc2, anti-cyclin B1, and all secondary antibody IgG-HRP conjugates (Santa Cruz Biotechnology, Santa Cruz, CA).

**Clonogenic Survival Assays.** Cell survival after treatment with 6-TG (Sigma Chemical Co.) or IR (Model 101.17 \( ^{13} \text{Cs} \)) irradiation at a dose rate of 4.1 Gy/min) was determined by a standard colony-forming assay. For 6-TG, 250 or 500 cells were plated into 60-mm dishes and allowed to adhere for 15 h. Cells were then treated with 6-TG for 24 h and subsequently incubated in fresh medium for 10–14 days for colony formation. For survival analyses after IR, cells were replated into 60-mm dishes at 250 or 500 cells/dish immediately after IR exposures and allowed to grow for 10–14 days to form colonies. The colonies were stained with 0.5% crystal violet in methanol/acetic acid (3:1), and those comprised of >50 cells were counted. Experiments were performed in medium without antibiotics, and all experiments were performed at least twice, each in duplicate.

**Synchronization and Cell Cycle Assays.** To determine cell cycle changes after 6-TG treatment, cells were seeded at 1–3 \( \times 10^5 \)–10^6/cm plate (cell number varied according to their sensitivity to 6-TG). 6-TG (3 \( \mu \text{M} \)) was then added −15 h after seeding, and the medium (with 6-TG) was changed every 2 days for a total 6-TG exposure of five to six population doubling times. For cell cycle analyses after IR treatment, cells were seeded at 1 \( \times 10^5 \)–10^6/cm plate and irradiated 24 h later. Cells were harvested at different times after IR according to their population doubling times. All cells were trypsinized, washed with PBS, and fixed in 95% ethanol/10% \( \mu \text{g/ml} \) of PBS. For PI staining, samples were washed with phosphate/citric acid buffer [192 mM Na\(_2\)HPO\(_4\) and 4 mM citric acid (pH 7.8)], resuspended in PI solution (1 mg/ml RNase A, 33 \( \mu \text{g/ml} \) in PBS, 0.5 mM EDTA, and 0.2% NP40) and then incubated at 4°C overnight. Flow cytometry analyses were 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 House, Topsham, ME). All experiments were repeated 2–4 times.

To determine IR-induced cell cycle changes in synchronous cells, synchronization of cells in G\(_0\)/G\(_1\) was carried out using contact inhibition/serum starvation. Briefly, cells were grown to confluence for ~35 h. Cells were then grown in medium containing 0.1% FBS for another ~35 h. Synchrony was achieved by trypsinization and cell replating at a low density of 1.5 \( \times 10^6 \) cells/cm\(^2\) plate in complete DMEM. The synchronized cell populations were irradiated at 2 h after replating when cells were still in G\(_0\)/G\(_1\) or at 16 h after replating when cells were in S phase. Cell cycle progression was determined at 4-h intervals for 3 days after replating. Similar techniques for cell preparation, staining, and flow cytometry at various times before or after IR were performed, as described above.

**Western Blot Analyses.** Cell lysates were prepared according to the nuclear extraction method (33), with recovery of both cytoplasmic and nuclear fractions. Quantitation of protein was performed using Bio-Rad protein assays (Bio-Rad Laboratories, Hercules, CA). Protein separation was performed by SDS-PAGE (9–12% polyacrylamide with 4% stacking gel), and separated proteins were then electrotransferred onto Immobilon-P membranes (Millipore, Danford, MA). Western blot analyses used 10% nonfat dry milk as a blocking solution, PBST (PBS with 0.2% Tween 20) as a washing buffer, various primary antibodies in 10% milk at 4°C overnight, and secondary antibodies in 5% milk at room temperature for 1 h. Results were visualized on X-ray film using SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL). All experiments were performed at least twice.

**Statistics.** All data in survival graphs represent means ± SE. Data were analyzed using Student’s \( t \) test.

**Results**

**Effect of hMLH1 Deficiency on Cell Survival and G\(_2\)-M Arrest after 6-TG or IR Treatments.** We reported previously that two different MLH1-deficient cell lines, human colorectal tumor HCT116 cells, and primary mlh1-null MEFs EF5, showed a reduced and shorter G\(_2\)-M arrest response after IR compared with their genetically matched MLH1-proficient cells (18). Two additional human tumor cell lines, A2780/CP70 and RKO, which also lack expression of the hMLH1 protein, were used in this study to confirm the role of MLH1 in an IR-induced G\(_2\)-M arrest. As depicted in Fig. 1A, we confirmed that the MMR-proficient CH3 cell line showed expression of the hMLH1 protein, whereas hMLH1-deficient cell lines lacked this repair protein. Expression of hPMS2, an important dimeric partner of hMLH1, was also noted in CH3 cells, indicative of functional hMLH1 that stabilizes the hPMS2 protein (34). Expression of hMSH2 protein was also monitored. hMSH2 protein was equally expressed among the three cell lines independent of hMLH1 status (Fig. 1A).

Responses of A2780/CP70 cell lines to 6-TG were used as a phenotype control of MMR-proficiency and -deficiency, as described previously (18). Exposure of MMR-deficient CP70 and CH2 cells to low concentrations of 6-TG (1.5–6 \( \mu \text{M} \)) resulted in higher clonogenic survival and reduced G\(_2\)-M arrest compared with MMR-proficient cells (Fig. 1, B and C). MMR-proficient CH3 cells showed the expected cytotoxic and G\(_2\)-M arrest responses to 6-TG, indicating that ecotopically expressed hMLH1 protein was functional.

Similar clonogenic survival assays and cell cycle analyses were performed using the A2780/CP70 cell system after IR exposures. As with HCT116 cells (18), hMLH1-deficient CP70 and CH2 cells displayed a reduced and shorter G\(_2\)-M arrest compared with MMR-proficient CH3 cells (Fig. 1E). In all three cell lines, treated cells arrested in G\(_2\)-M with peak levels at 12 h after 6 Gy. However, hMLH1-deficient CP70 and CH2 cells arrested at the G\(_2\)-M transition point to a lesser extent than hMLH1-proficient CH3 cells. Furthermore, analyses at 25 h after IR showed that hMLH1-deficient CP70 and CH2 cells exited G\(_2\)-M arrest more rapidly than hMLH1-proficient CH3 cells. Finally at 36 h, the G\(_2\)-M fraction of IR-treated hMLH1-deficient cells decreased to ~25% of total cell population, whereas ~46% of IR-treated hMLH1-proficient CH3 cells remained in G\(_2\)-M phase. Interestingly, clonogenic survival after high-dose-rate IR did not differ significantly among the three cell lines with or without hMLH1 expression (Fig. 1D), in contrast with the results observed after...
6-TG treatment (Fig. 1B). These results are similar to our previously published data using HCT116 and mlh1-null MEF cell systems (18).

Loss of hMLH1 expression may also occur by epigenetic hypermethylation of its promoter. To explore the sensitivity of such hMLH1-deficient cells that stably reexpress the protein after 5-deoxyazacytidine exposure, we examined the response of the RKO cell system to IR or 6-TG. Western blot analyses of hMLH1 and hPMS2 proteins in hMLH1-proficient RKO/aza50 cells (Fig. 2A). Similarly, hMLH1-deficient RKO cells demonstrated a reduced and shorter G2-M arrest compared with HEC59/2-4 cells (Fig. 2D). The hMLH1-deficient RKO cells also showed a modest but not significant increased clonogenic survival after IR (Fig. 2D).

Effect of hMSH2 Deficiency on Cell Survival and G2-M Arrest after 6-TG or IR Exposures. hMSH2-deficient and -proficient HEC59 cells were next used to determine whether MMR-deficiency, in general, affects G2-M arrest after IR. As expected, MMR-corrected HEC59/2-4 cells expressed both hMSH2 and hMSH6 proteins (Fig. 3A), presumably by expression from the transferred human chromosome 2. There was no difference in hMLH1 expression between the two cell lines (Fig. 3A).

In contrast with hMSH2-expressing HEC59/2-4 cells, MMR-deficient HEC59 cells showed higher clonogenic survival and a markedly reduced G2-M arrest response to 6-TG treatment (Fig. 3, B and C). As with the isogenetically matched hMLH1 human tumor cell systems, no significant difference in clonogenic survival was found between HEC59 and HEC59/2-4 cells after IR (Fig. 3D).Interestingly, the cell cycle response after IR demonstrated that, similar to responses in hMLH1-deficient cell lines, hMSH2-deficient HEC59 cells exhibited a reduced and shorter G2-M arrest compared with HEC59/2-4 cells (Fig. 3E). On the basis of these data in hMLH1 and hMSH2 human tumor cell systems, we suggest that MMR affects the G2-M arrest responses after high-dose-rate IR damage without clear differences in clonogenic survival. In contrast, MMR influenced both the G2-M arrest and cytotoxic responses to 6-TG treatment.

Differences in the IR-induced G2-M Arrest between MMR-proficient and -deficient Cells Are Not Associated with Different G1 Arrest and/or S-phase Delay Responses. We also investigated whether our observed differences in the IR-induced G2-M arrest between MMR-proficient and -deficient cells were associated with differences in a G1 arrest or an S-phase delay. We addressed this question using synchronized RKO and RKO/aza50 cells because the other two human tumor cell systems, CP70 and HEC59, lacked a clear G1 arrest after IR in preliminary experiments, which may relate to p53 status. The RKO system is p53 wild-type, whereas the HEC59 system is p53 mutant, and the CP70 system has a wild-type p53 DNA sequence but the p53 protein is not functional (35). RKO and RKO/aza50 cells were synchronized in G0/G1 by growth to confluence and then serum starvation (0.1% serum in complete medium) as described. The G0/G1 synchronized cells were then released by replating at low density in complete medium (with 10% FBS) and irradiated with 6 Gy at two separate time points: at 2 h after release (when the majority of the cells were still in G0/G1 phase) or at 16 h after release (when the majority of the cells were in S phase). Cell cycle progression was determined by flow cytometry at 4-h intervals. Fig. 4 shows the proportion of G2-M cells at each time point after release.
in the control (Fig. 4A) and the irradiated samples (Fig. 4B and 4C). When synchronized cells were exposed to IR in S phase (16 h postrelease), the peak population of cells in G2-M phase were equivalent in both cell lines at 14 h post-IR (Fig. 4C). These suggest that the cell lines had undergone a similar S-phase progress after IR irrespective of MMR status. However, a significant difference in the G2-M fraction was noted at 22 h after IR, with 20% more G2-M cells in MMR-proficient RKO/aza50 cells than in MMR-deficient RKO cells (Fig. 4C). This difference was sustained for up to ~40 h.

When the cells were irradiated in G0/G1 phase (2 h postrelease), we noted comparable G2-M peaks between the two cell lines at 24 h post-IR (Fig. 4B), suggesting a similar G1 phase progress between the two cell lines. Moreover, only a small and transient (about 12-h) difference in G2-M arrest between MMR-deficient RKO and -proficient RKO/aza50 cells was observed after the G2-M peaks when G0/G1 cells were irradiated (Fig. 4B). The peaks of the G2-M arrest in both cell lines were found to be lower when IR was delivered to G0/G1 phase synchronous cells (Fig. 4B) than that when IR was delivered to S phase-synchronous cells (Fig. 4C). These data suggest that DNA damage sensed by MMR have been partially repaired before cells approached the G2-M checkpoint.

MMR-deficient Cells Show Reduced Tyrosine 15-Phosphorylation of cdc2 during G2-M Arrest. We next sought to characterize further a possible signal transduction pathway affecting the G2-M delay after IR damage in MMR-deficient and -proficient cells. IR-induced G2-M arrest responses have been associated with the cdc2 pathway (21, 36). As a key regulator of the G2-M transition, cdc2 phosphorylation is indicative of G2 arrest. Dephosphorylation of cdc2 at threonine 14 and tyrosine 15 activates cdc2 kinase activity resulting in entry into M phase (37, 38). We, therefore, examined the phospho-Tyr15 status of the cdc2 protein in hMLH1-deficient CP70 and -proficient CH3 cell lines, as well as in hMSH2-deficient HESC9 and -proficient HESC9/2-4 cell lines. Cells were irradiated with 5 Gy and harvested at 12 and 24 h post-IR for CP70 cells, and at 24 and 48 h post-IR for HESC9 cells. These time points were selected based on the G2-M arrest responses shown in Fig. 1E and 3E, respectively. Western blots were used to assess phospho-Tyr15-cdc2 levels (Fig. 5A). Phospho-Tyr15-cdc2 levels increased significantly after IR in all cell lines, and higher phospho-Tyr15-cdc2 levels were noted in the MMR-proficient CH3 and HESC9/2-4 cells, corresponding to the greater G2 arrest responses (Figs. 1E and 3E). Additionally, phospho-Tyr15-cdc2 levels decreased more rapidly in the hMLH1-deficient CP70 cells and the hMSH2-deficient HESC9 cells, compared with the hMLH1-proficient CH3 cells and the hMSH2-proficient HESC9/2-4 cells. Interestingly, total cellular levels of cdc2 resembled to follow cdc2 phosphorylation, Where elevated levels were noted in MMR-proficient cells. Only transient and small increases of cdc2 were noted in MMR-deficient cells. The levels of cyclin B1, a dimeric partner of cdc2 kinase complex, increased and decreased in a manner similar to that of phospho-Tyr15-cdc2. Elevated levels of cyclin B1 as well as elevated levels of phosphorylated cdc2 strongly indicate a G2 arrest response instead of a mitotic arrest. These responses are in agreement with our recently published data comparing the cytotoxic and cell cycle responses to 5-fluoro-2'-deoxyuridine treatment in MMR-deficient versus MMR-proficient cells, wherein lack of MPM-2 and elevated cyclin B and cdc2 phosphorylation strongly suggested G2 arrest and not a mitotic delay (39). Collectively, the data suggest that MMR may participate directly or indirectly in the regulation of G2 cell cycle checkpoint arrest through the strong phosphorylation of cdc2 protein following IR damage.

Because MMR-proficient cells show a robust G2-M arrest in response to 6-TG treatment, we also examined whether the 6-TG-
induced G2-M arrest is associated with increased phosphorylation of cdc2 protein. Western blot analyses were carried out in similar cell populations that were treated with 3 μM 6-TG for five to six cell population doubling times. As presented in Fig. 5B, both phospho-Tyr15-cdc2 and cyclin B1 were increased significantly in the MMR-proficient CH3 and HEC59/2-4 cells, but not in the MMR-deficient CP70 and HEC59 cells, corresponding to their G2 arrest status (Fig. 1C and 3C). These data suggest that the G2 arrest after 6-TG genotoxic stress is also mediated through cdc2 signaling. Although it seems that the cdc2 pathway participates in the G2 arrest response after both IR and 6-TG, other downstream events must be different, as we have shown that MMR-mediated, 6-TG-induced G2 arrest is associated with cell death, whereas MMR-mediated, IR-induced G2 arrest does not significantly affect cell survival (Figs. 1–3).

DISCUSSION

DNA damage-induced G2-M cell cycle checkpoint arrest response is a complex process (40–42). Depending upon the type of damaging agent used, the same cell may show a completely different G2-M arrest response (40, 41). IR is well known to trigger a G2 arrest through multiple mechanisms. The ATM/ATR-cdc2 pathway is a key regulator (21, 22). However, p53/p21 (23, 24), GADD45 (25), hRAD9 (26), mitogen-activated protein kinase (27), BRCA1 (28), and RINT-1 (29) are also reported to participate in the regulation of an IR-induced G2 arrest. In this paper, we show that the cdc2 pathway participates in the G2 arrest response after both IR and 6-TG, other downstream events must be different, as we have shown that MMR-mediated, 6-TG-induced G2 arrest is associated with cell death, whereas MMR-mediated, IR-induced G2 arrest does not significantly affect cell survival (Figs. 1–3).

we extend our initial findings to two additional hMLH1-deficient human tumor cell lines, A2780/CP70 and RKO. The new data confirm our previous finding that the expression of MLH1 protein results in a proficient IR-induced G2 arrest, which is higher and more sustained compared with the G2 arrest in the genetically matched MLH1-deficient cells. Because these four MLH1-deficient cell model systems differ in cell type (i.e., mouse and different human tumor origins), p53 status, mechanism of MLH1 deficiency (i.e., genetic and epigenetic silencing), and the approach used to restore hMLH1 activity (human chromosome 3 transfer or 5-deoxyazacytidine treatment for demethylation), our combined data strongly suggest that the observed reduced and shorter G2 arrest responses to high-dose-rate IR are a general characteristic of MLH1-deficiency. In our previous study (18), we also examined the role of p53 in this altered G2 arrest by knocking-out p53 function with E6 protein in the hMLH1-deficient HCT116 and the hMLH1-proficient HCT116/3-6 lines. The presence or absence of p53 protein did not affect the IR-induced G2 arrest in the HCT116 cell system, and our current data confirm these results inasmuch as CP70 cells have no functional p53 (35), and RKO cells have wild-type p53 (43).

We also examined hMSH2-deficient human tumor HEC59 cells to determine whether hMSH2 had any effect on G2-M arrest after IR or 6-TG treatment. We found that hMSH2-deficient HEC59 cells showed the classic damage-tolerance responses to 6-TG treatment, manifested by reduced cytotoxicity and G2 arrest responses compared with genetically matched hMSH2-expressing HEC59/2-4 cells (Fig. 3, B and C). hMSH2-deficient HEC59 cells also showed a reduced and shorter G2 arrest after IR (Fig. 3E), but no significant difference in cytotoxicity compared with HEC59/2-4 cells (Fig. 3D). Thus, deficiency in either of the critical proteins in functional MMR impairs the...
IR-induced G2 arrest, and therefore the MMR system in general seems to be a key regulator of G2 arrest responses. Notably, in all five cell systems we studied, MMR-deficiency only partially abrogates the G2 arrest after IR; the difference is 10–25% of the total cell population. This observation agrees with the notion that the IR-induced G2 arrest is regulated by multiple mechanisms.

Using synchronized cell populations, we attempted to assess any effect of other cell cycle checkpoint responses (i.e., G1 or S-phase) on our observed differential G2 arrest after IR in MMR-deficient versus MMR-proficient cells. There are several interesting observations. First, the difference in an IR-induced G2 arrest between MMR-proficient and -deficient cells occurred regardless of whether cells were irradiated in G0/G1 (Fig. 4B) or S phase (Fig. 4C), indicating that MMR indeed has an impact on the G2/M checkpoint arrest. Second, the differences in the IR-induced G2 arrest were smaller and more transient when IR was given to synchronized G0/G1 phase cells than that when IR was given to synchronized S-phase cells, implying that the IR damage sensed by MMR has been partially repaired during the G1 arrest and S delay. Third, the differences in IR-induced G2 arrest between MMR-proficient and -deficient cells appeared only after reaching equal G2 peaks, suggesting a slower exit of MMR-proficient cells from G2 arrest.

IR induces a spectrum of DNA damage including DSBs, single strand breaks, cross-links, and oxidative base modification (44). It has been suggested that MMR may be principally involved in the repair of the oxidative-modified bases after IR (45). In this regard, it has been shown that a major oxidative product, 8-oxoguanine, can mispair with adenine during DNA replication and increasing evidence suggests that MMR recognizes and removes this mispair (46–48). According to this hypothesis, abortive MMR or collisions between repair and replication machinery result in the production of DSBs. This MMR-dependent production of DSBs may then be the key signal that triggers ATM/ATR-dependent G2 arrest responses. However, one would anticipate that such a mechanism would lead to more cell death in MMR-proficient cells, which we did not find after high-dose-rate IR. Another potential mechanism could be that MMR proofreads DNA base mispairs as well as loops generated by DNA nonhomologous end joining, which is known to be the major repair pathway after IR. Our speculation is that the MMR system senses certain forms of DNA damage and mediates the cdc2 signaling pathway in response to IR. Consistent with this proposed role of MMR are our findings that both hMLH1 and hMSH2 proteins are present constitutively at relatively high levels throughout the cell cycle (49) and are not induced by IR (18).

The ultimate goal of the G2 checkpoint cdc2 pathway is down-regulation of cdc2 kinase activity through increased cdc2 phosphorylation, enabling cells to repair lesions before entering M phase (50). In this study, we provide the first evidence that tyrosine15-phosphorylation of cdc2 differs in MMR (hMLH1 or hMSH2)-deficient versus -proficient cells subsequent to IR. Early after IR, increased levels of phospho-Tyr15-cdc2 were noted in both MMR-proficient and -deficient cell lines, corresponding with the peak G2 arrest responses. It is known that IR directly induces DSBs, which may stimulate ATM/ATR and could result in a MMR-independent G2 arrest. However, the levels of phospho-Tyr15-cdc2 in the MMR-deficient cell lines were transient and correlated temporally with the transient G2 arrest responses observed. In contrast, in MMR-proficient cells, levels of phospho-Tyr15-cdc2 remained high, corresponding to the slow release of IR-damaged cells from G2 arrest. On the basis of these observations, we conclude that MMR plays a major role in regulating cdc2 phosphorylation and G2 arrest in response to IR.
observations, we speculate that the MMR system might mediate an IR-induced G2 arrest by interacting with other proteins which activate the cdc2 signaling pathway, or MMR might interact directly with one or more components of cdc2 signaling pathway. Alternatively, MMR may indirectly induce a G2 arrest through a proofreading response that generates DSBs which in turn trigger ATM/ATR- or DNA-PK-dependent G2 arrest responses.

Support for a more direct role of MMR in G2 arrest may be gleaned from data in *Escherichia coli*, where the MutL, NH2 terminus is structurally homologous to DNA gyrase, class II DNA topoisomerases, histidine kinase, and Hsp90 protein, and MutL has a conserved ATP binding site and weak ATPase activity, similar to Hsp90 (51, 52). These findings suggest that MutL may act as a molecular chaperone. MutL is also known to interact with the DNA helicase UvrD (53, 54), and plays a role in recruiting DNA polymerase III to complete the repair process after MutH nicks the daughter strand (2).

In human cells, hMLH1 has been found to interact with MutSα and PCNA (55). Ducottet et al. (56) reported the hMutLα-dependent activation of one or more protein kinases that phosphorylate the p53 tumor-suppressor protein in response to DNA methylation damage. Yang (57) proposed that MutL may be a molecular switch that recruits different proteins at various steps in the MMR process. The potential involvement of MutS and MutL and their homologues in both repair of oxidative or carcinogen-damaged DNA and programmed cell death responses strongly suggest the requirement of a molecular switch to coordinate various DNA repair pathways and to choose between repair versus apoptosis (57). MutL seems to be a promising candidate to fulfill such a requirement. Although hMSH2 has also been reported to interact with other proteins, the structure of the hMSH2 protein seems to be limited in its ability to bind a broader protein spectrum.

We speculate that hMSH2 (MutS) might sense certain IR-induced DNA damage and hMLH1 (MutL) might relay this signal to other proteins that regulate the cdc2 pathway. The presence of both hMLH1 and hMSH2 proteins might be a prerequisite to deliver an IR-induced DNA damage signal to the cdc2 pathway. In other words, IR-induced DNA damage may not be sensed without hMSH2 (MutS), and the signal could not be relayed to the cdc2 pathway without the hMLH1 (MutL). The mechanism of MMR-dependent G2 arrest remains unknown, and we are currently exploring whether MMR directly signals cdc2 phosphorylation via stimulation of ATM/ATR-cdc2, or whether MMR generates DSBs during repair of IR or 6-TG-induced DNA damage, which in turn triggers ATM/ATR-cdc2 responses.

Interestingly, MMR-proficient and -deficient human tumor cells used in this study and in our previous study (18) demonstrated little, if any, differences in clonogenic survival after high-dose-rate IR. Similar results were found by several other investigators. Leardon et al. (58) reported that deficiencies in either hMSH2 or hMLH1 did not lead to an increased sensitivity to IR in HCT116 (hMLH1−) and HCT116/3-6 (hMLH1−), or HEC59 (hMSH2−) and HEC59/2-4 (hMSH2−) cells. Likewise, O’Driscol et al. (59) found no significant change in cell killing by IR in hMSH2-deficient xeroderma pigmentosum cells, and Aquilina et al. (60) demonstrated that a hPMS2 deficiency did not alter cell survival to IR in HeLa cells. However, Fritzell et al. found a small but statistically significant increase in survival after high-dose-rate IR in murine MMR-deficient cell lines (45). Additionally, low-dose-rate IR exposures to MMR-deficient murine cells led to significantly increased survival compared with MMR-proficient cells (48, 61). The exact mechanism(s) of why low-dose-rate IR exposures cause a greater MMR-dependent survival difference compared with high-dose-rate IR is not known. However, we speculate that the differences in G2 arrest between MMR-deficient and -proficient cells may be a major factor in these differential survival responses after low-dose-rate IR versus high-dose-rate IR. Because G2 arrest is a highly sensitive phase of the cell cycle, a longer MMR-dependent G2 arrest during continuous low-dose-rate IR of MMR-proficient cells would greatly decrease their survival. Alternatively, MMR-deficient cells would sustain less low-dose-rate IR damage during a shorter G2 arrest and then would differentially arrest in the more radioresistant G1 phase of the cell cycle, thus affording a survival advantage, albeit with greatly increased mutation rates.

Because the impaired G2 arrest in MMR-deficient cells was not accompanied by a marked change in cell survival in our studies, a question arises regarding the biological significance of the role of MMR in the IR damage response. As mentioned, a general belief is that a proficient G2 arrest allows cells time to repair DNA damage before mitosis, presumably enhancing cell survival with reduced mutations after exposures to DNA damaging agents. Cells that have lost the ability to arrest in G2 subsequently die. For example, interruption of G2 arrest by caffeine has long been known to result in enhanced IR-induced cell killing (62). Interestingly, though MMR-deficient cells have a defective G2 arrest response, they demonstrated no significant differences in survival compared with MMR-proficient cells. One possible explanation is that cell death from IR is primarily attributable to DSBs that presumably cause mitosis-linked loss of replicative ability and apoptosis (63). Therefore, we hypothesize that the biological significance of MMR in IR-induced G2-M arrest might be to protect the cell from IR-induced mutations. The fact that the initial G2 arrest responses are comparable in MMR-proficient and -deficient cells and that half of the IR-induced DSBs are repaired within ~2 h post-IR may explain the lack of a survival response between these two genetically matched cell populations. The apparent difference in G2 arrest between MMR-proficient and -deficient cells may be caused by a lack of proofreading after IR, leading to transient signaling in MMR-deficient cells and, in contrast, sustained proofreading and signaling in MMR-proficient cells.

In summary, our results confirm our previous observation that hMLH1-deficiency results in a reduced and shorter G2 arrest response after IR. Similar responses were found in hMSH2-deficient cells. Therefore, the data strongly suggest that the MMR system plays a role in the regulation of G2 arrest after IR. Our results with synchronized cells ruled out a possible contribution of G1 arrest and S-phase delay to the observed difference in IR-induced G2 arrest. We also provide the first evidence that the magnitude and duration of phosphorylation of cdc2 protein is diminished after IR in MMR-deficient versus MMR-proficient cells. Difference in phosphorylation of cdc2 correlated well with the observed time course of the G2 arrest response, implying that MMR proteins may mediate the regulation of the cdc2 signaling pathway in response to IR. These data raise the possibility that MMR participates directly in the regulation of the G2-M cell cycle checkpoint after IR, possibly controlling overall mutation rates in exposed cells.

REFERENCES
Loss of DNA Mismatch Repair Imparts Defective cdc2 Signaling and G2 Arrest Responses without Altering Survival after Ionizing Radiation

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