Defective Expression of the DNA Mismatch Repair Protein, MLH1, Alters G2-M Cell Cycle Checkpoint Arrest following Ionizing Radiation

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ABSTRACT

A role for the Mll homologue-1 (MLH1) protein, a necessary component of DNA mismatch repair (MMR), in G2-M cell cycle checkpoint arrest after 6-thioguanine (6-TG) exposure was suggested previously. A potential role for MLH1 in G1 arrest and/or G2-S transition after damage was, however, not discounted. We report that MLH1-deficient human colon carcinoma (HCT116) cells showed decreased survival and a concomitant deficiency in G2-M cell cycle checkpoint arrest after ionizing radiation (IR) compared with genetically matched, MMR-corrected human colon carcinoma (HCT116 3-6) cells. Similar responses were noted between murine MLH1 knockout compared to wild-type embryonic fibroblasts. MMR-deficient HCT116 cells or embryonic fibroblasts from MLH1 knockout mice also demonstrated classic DNA damage tolerance responses after 6-TG exposure. Interestingly, an enhanced p53 protein induction response was observed in HCT116 3-6 (MLH1+) compared with HCT116 (MLH1-) cells after IR or 6-TG. Retroviral vector-mediated expression of the E6 protein did not, however, affect the enhanced G2-M cell cycle arrest observed in HCT116 3-6 compared with MLH1-deficient HCT116 cells. A role for MLH1 in G2-M cell cycle checkpoint control, without alteration in G1, after IR was also suggested by similar S-phase progression between irradiated MLH1-deficient and MLH1-proficient human or murine cells. Introduction of a nocardazole-induced G2-M block, which corrected the MLH1-mediated G2-M arrest deficiency in HCT116 cells, clearly demonstrated that HCT116 and HCT116 3-6 cells did not differ in G1 arrest or G2-S cell cycle transition after IR. Thus, our data indicate that MLH1 does not play a major role in G1 cell cycle transition or arrest. We also show that human MLH1 and MSH2 steady-state protein levels did not vary with damage or cell cycle changes caused by IR or 6-TG. MLH1-mediated G2-M cell cycle delay (caused by either MMR proofreading of DNA lesions or by a direct function of the MLH1 protein in cell cycle arrest) may be important for DNA damage detection and repair prior to chromosome segregation to eliminate carcinogenic lesions (possibly brought on by misrepair) in daughter cells.

INTRODUCTION

Hereditary nonpolyposis colorectal cancer is a familial colon cancer syndrome that accounts for 1-5% of all colorectal tumors (1). This syndrome is caused by inherited mutations in several MMR genes, including MSH2, MLH1, and PMS2 (2-6). Mutations within these genes have also been observed in sporadic cancers of various origins (7-10). The MMR system is important for maintaining genomic fidelity through the recognition and repair of incorrectly paired nucleotides, which can arise in DNA by physical damage to existing nucleotides, DNA polymerase incorporation errors, or heteroduplex formation during genetic recombinational events (11-14). Expression of the MLH1 gene, mapped to chromosome 3p21, is essential for competent MMR and maintenance of microsatellite stability (15, 16). Because MLH1 has no known enzymatic activity or functional motifs, it has been hypothesized to play a role as a "molecular matchmaker" for other DNA repair proteins (17). Transgenic mice lacking MLH1 expression develop normally but present with lymphomas at an early age and are sterile, presumably due to faulty meiotic chromosome segregation (18, 19). To date, the only data on functional activity in mammalian cells suggest that the MLH1 protein may regulate G2-M cell cycle checkpoint arrest following 6-TG or MNNG exposure, which results in DNA base damage and mispaired lesions (20).

Previously, Hawn et al. (20) and Hawn et al. (21) generated and characterized a genetically matched human colon carcinoma model system, HCT116 (MLH1-) and HCT116 3-6 (MLH1+). HCT116 cells were deficient in DNA MMR (22) due to the lack of MLH1 transcript expression (4, 5). HCT116 3-6 (MLH1+) cells were generated by introducing (via microcell fusion) a single copy of normal human chromosome 3 into HCT116 cells (21). These corrected cells demonstrated expression of the MLH1 transcript (by single-strand conformation polymorphism-PCR), reconstituted MMR, increased stability in the maintenance of lengths of microsatellite sequences, and an increased sensitivity to MNNG or 6-TG (20, 21). HCT116 cells were apparently defective in the G2-M cell cycle arrest checkpoint when compared with the MMR-competent, HCT116 3-6 cells after 6-TG exposure (20). These previous data did not, however, eliminate a role for the MLH1 protein in G1 arrest or transition from G1 into S phase.

In general, the mechanisms of damage-induced G2-M cell cycle checkpoint arrest in mammalian cells are poorly understood. A number of regulatory proteins have been implicated in the G2-M cell cycle checkpoint, including cyclin B1, lyn kinase, cdc2, cdc25, and topoisomerase II-α (23-30). We, therefore, sought to determine whether the MLH1 protein, and MMR in general, played a role in G2-M cell cycle checkpoint responses following other forms of DNA damage, such as IR. The role of MMR in processing damage caused by IR, compared with agents that directly cause base mispairing (e.g., 6-TG), is not known. IR treatment generates a myriad of DNA lesions, including the formation of modified nucleotides, abasic sites, DNA-protein cross-links, SSBs, and DSBs (31, 32). The most lethal of these lesions are thought to be DSBs (33). DNA mismatches may arise directly from IR treatment or may result from later DNA repair...
processes that are error prone (31, 34). By examining survival, protein expression, and cell cycle distribution, we have determined a role for MLH1 in the cellular responses to IR damage compared with those induced by 6-TG, which incorporates into DNA during replication (35, 36) and is recognized by the MMR machinery (20).

To determine the specific role of the MLH1 protein in damage-inducible G2-M cell cycle checkpoint arrest, as well as other cellular responses to IR or 6-TG, we used the genetically matched HCT116/6-TG cell system, along with genetically matched primary embryonic fibroblasts from transgenic MLH1 knockout, heterozygous, or wild-type siblings. We also provide evidence suggesting that MLH1-mediated G2-M cell cycle checkpoint control is p53 independent and that MLH1 function does not influence, nor is influenced by, p53-mediated G1 cell cycle checkpoint control, which has been extensively investigated following IR (37–40).

MATERIALS AND METHODS

Chemicals, Cell Culture, Cell Treatments, and Survival Determinations. The HCT116 + human chromosome 3, clone 6 (HCT116-3-6), cell line was created by microcell chromosome transfer of a single normal human chromosome 3 (tagged with pSV2-neo plasmid DNA) into HCT116 human colon carcinoma cells (21). Parental HCT116 cells have a hemizygous nonsense mutation in the MLH1 gene located on chromosome 3 (5, 41). HCT116 cells, which were microcell fused and selected for the retention of human chromosome 2 (HCT116-2-1), were also used as controls. Because HCT116-2-1 cells had identical survival (Fig. 2), cell cycle responses and p53 induction characteristics to HCT116 (data not shown), we only present data from HCT116 and HCT116-3-6 cells. The doubling times of HCT116, HCT116-3-6, and HCT116-2-1 cells were not statistically different and were calculated to be 18 ± 1.2, 20 ± 2.3 h, and 20 ± 2.0 h, respectively.

Primary embryonic fibroblasts were prepared as described (42). Transgenic (C57BL/6 background) MLH1+/+ heterozygous mice (generously provided by Dr. R. M. Liskay at Oregon Health Science University, Portland, OR) were mated, and fetuses were isolated from 13- to 15-day pregnant females. Primary embryonic fibroblast cultures were derived by dissociating individual embryos with 0.05% trypsin in 0.53 mM EDTA (Life Technologies, Inc., Gaithersburg, MD) and plating the resulting individual cells on plastic tissue culture dishes. Fibroblast cultures from at least three embryos of each genotype [wild-type (MLH1+/+), heterozygous (MLH1+/−), or homozygous (MLH1−/−)] were examined after genotyping via specific PCR primers and standard PCR amplification techniques for MLH1 gene analyses as described (19). Growth of wild-type, heterozygous, and homozygous primary embryonic fibroblasts were essentially identical, and doubling times were estimated to be 24 ± 2 h. Data from representative cultures, EF37 (+/−), EF19 (+/−), and EF5 (−/−), were shown.

Both HCT116 cells and primary embryonic fibroblasts from murine MLH1 knockout animals, as well as from their corresponding wild-type siblings, were maintained in DMEM (Life Technologies, Inc.), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin and grown in a 90% air-10% CO2 atmosphere at 37°C. Cells were typically grown to 50% confluency at the start of each IR experiment and 10–20% confluency at the start of each 6-TG experiment. 6-TG (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.1 N NaOH. X-ray treatments were performed with a Model 109 137Cs irradiator (J. L. Shepherd and Associates, Glendale, CA) at a dose rate of 6.1 Gy/min. For 6-TG experiments, cells were grown in modified a-MEM lacking ribonucleosides and deoxyribonucleosides (Life Technologies, Inc.) with 10% FBS for 5 days to allow sufficient 6-TG uptake. The surviving fraction of cells after IR or 6-TG treatment was determined by serial dilution colony-forming ability assays as described (43); cells were replated immediately after IR treatment, whereas 6-TG survival assessments were performed after 24-h drug exposures. As described previously, only colonies with ≥50 normal-appearing cells were counted and scored as a viable colony (44). SERs were calculated to compare MMR-proficient versus MMR-deficient cells after IR. All survival experiments were performed at least five times, each in duplicate. All data points represent means ± SE, and survival curves were compared using Student’s t test.

Chemically Induced G2-M Arrest of HCT116 and HCT116-3-6 Cells. Cells were chemically arrested in G2-M using the microtubule inhibitor nocodazole (40 nm; Sigma), which was added immediately after IR exposure. At a dose of 40 nm, nocodazole was reversibly cytostatic, with little or no cytotoxicity. Control conditions included no treatment, nocodazole alone, and IR alone. All nocodazole experiments described were performed at least three times, each in duplicate. All data points represent means ± SE.

Generation of HCT116 and HCT116-3-6 Cell Lines Expressing Papillomavirus E6. HCT116 or HCT116-3-6 cells (5 × 105) were plated in 10-cm2 dishes and allowed to attach overnight. Cells were then exposed for 4 h to a retroviral vector containing or not containing (vector only) the HPV16 E6 HYGRO mammalian expression vector in the presence of 4 μg/ml Polybrene (to enhance viral attachment to the membrane). Fresh medium was then added, and cells were allowed to recover and grow for 3 days. Cells were then seeded at low density and selected for hygromycin-resistance using 80 μg/ml hygromycin B (Boehringer Mannheim, Indianapolis, IN) in DMEM. After 2 weeks, colonies from individual cells were isolated and characterized for p53 expression and cell cycle arrest following IR or 6-TG treatments in the absence of hygromycin; cells were maintained in hygromycin, but all experiments were performed in the absence of this antibiotic. Although data from only one or two clones were presented, five or more clones of each cell type were investigated, each demonstrating similar responses. Fig. 8 shows the absence of inducible p53 in representative clones by Western analyses. Experiments using E6-transfected cells were performed at least three times, each in duplicate. All graphs, data points present means ± SE, and data sets were compared using Student’s t test.

Whole-Cell and Nuclear Protein Extraction and Western Immunoblot Analyses. To prepare nuclear extracts, cultured cells were scrapped into ice-cold PBS, swollen with an hypotonic solution [10 mM Tris (pH 7.5), 25 mM KCl, 2.45 mM magnesium acetate and 1 mM DTT], and lysed by 10 passages through a 25-gauge needle. Nuclei were pelleted by centrifugation (735 × g) and incubated in an hypertonic solution [10 mM Tris (pH 7.5), 400 mM KCl, 0.8 mM magnesium acetate, 0.4 mM dithiothreitol, 1 mM phenylmethylsulfonfluoride, and 20% glycerol; Ref. 45] and then prepared for Western blot analyses. For whole-cell extracts, cells were washed twice with ice-cold PBS, scraped, and pelleted by centrifugation (500 × g). Cell pellets were then lysed in standard RIPA buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1.0% NP40 (Sigma), 0.5% sodium deoxycholate, and 0.1% SDS) and prepared for Western blot analyses. Protein concentrations for nuclear and whole-cell samples were then determined by Bradford assays (46), 5 μg (nuclear) or 50 μg (whole cell) of each extract were fractionated by SDS-PAGE, and electrophoretically separated proteins were then transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore) as described (47, 48). Polyclonal antibodies to MLH1 (developed by us) or MSH2 (kindly provided by N. de Wind, Netherlands Cancer institute, Amsterdam, The Netherlands) were used at a dilution of 1:2500, and the DO-1 p53 monoclonal antibody was used at a dilution of 1:1000 as per the manufacturer’s instructions (Santa Cruz Biotechnology, Santa Cruz, CA). MLH1, MSH2, p53, and α-tubulin (Oncogene Science, used as a loading control) steady-state protein levels were detected using horseradish peroxidase-conjugated secondary antibodies (except for p53, which was horseradish peroxidase was already conjugated) and enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL) following the manufacturer’s instructions. All Western blot experiments were repeated five or more times, and the blots shown are representative of all data obtained.

Cell Cycle Analyses. Trypsinized cells were washed in ice-cold Tris-saline (10 mM Tris (pH 7.0), 50 mM NaCl), fixed in 90% ethanol, and stored for up to 1 week at 4°C. Cells were washed with phosphate-citric acid buffer (200 mM Na2HPO4, 100 mM citric acid (pH 7.8)) and stained with a solution containing 0.2% NP40 (Sigma), 7000 units/ml RNase A (Sigma), and 33 mg/ml pro-pidium iodide (Sigma) at 37°C for 10 min or 4°C overnight. Stained nuclei were then analyzed for the percentage of G2/M, G2, and G1/M-arrested cells by flow cytometry with a Beckton Dickinson FACScan (San Jose, CA) at a laser setting of 15.2 mW and an excitation wavelength of 488 nm. Data were analyzed using ModFit LT version 1.01 software (Verity Software House, Topsham, ME). All flow cytometry experiments were repeated five or more times, each in duplicate. Data shown indicate representative histograms or are a combination of
repeated experiments. Where applicable, data from two separately treated conditions were statistically analyzed using Student's t test, and samples were compared based on Ps.

RESULTS

MLH1 Expression before and after IR. We initially investigated whether specific proteins involved in MMR (i.e., MSH2 or MLH1) were damage inducible. As expected, the MLH1 protein was not expressed in parental HCT116 (MLH1−) cells but was expressed in HCT116 3-6 (MLH1+) cells (Fig. 1, see "c" control lanes). Further comparisons showed that the relative expression of MLH1 (i.e., MLH1:MSH2 ratio) in HCT116 3-6 cells was quantitatively similar to normal IMR-90 human fibroblasts (48). MSH2 protein levels were not altered by chromosome 3 microcell transfer and subsequent selection, since similar levels were expressed in both HCT116 (MLH1−) and HCT116 3-6 (MLH1+) cells (Fig. 1), and levels of MSH2 in either cell line were comparable to those in IMR-90 normal human fibroblasts (48).

We then examined the regulation of MLH1 and MSH2 proteins after IR. No alterations in the steady-state levels of MLH1 (M, 84,000 protein; Fig. 1) or MSH2 (M, 105,000 protein; Fig. 1) proteins were observed at 4 or 12 h after 2, 5, and 8 Gy in HCT116 (MLH1−) or HCT116 3-6 (MLH1+) cells. Additional experiments revealed no alterations in MLH1 or MSH2 levels for up to 24 h after irradiation (data not shown). Levels of α-tubulin were used as loading controls, because the steady-state levels of this protein were not altered after IR (49). These data suggest that MLH1 and MSH2 proteins were not altered by IR in either cell line. The proteins also do not appear to be cell cycle regulated in HCT116 colon cancer cells, because significant changes in cell cycle distribution occurred over the course of the experiment after various doses of IR (Figs. 3–5). These data are consistent with our recent cell cycle analyses of synchronized normal human IMR-90 fibroblast cells in which MLH1 and MSH2 protein and transcript levels remained constitutively expressed during cell cycle progression (48).

Effect of MLH1 Expression Deficiency on Survival after IR or 6-TG. We then examined differences in colony-forming ability between HCT116 and HCT116 3-6 cells after IR (0–6 Gy). A statistically significant difference in survival (P < 0.049; Ref. 43) between HCT116 and HCT116 3-6 cells was found, with the SER of the former being modestly (1.46-fold at LD50) more sensitive to IR damage (Fig 2A). HCT116 2-1 cells, which contained an irrelevant chromosome (no. 2), showed equivalent survival to HCT116 cells. We also examined the survival differences between HCT116 and HCT116 3-6 cells after 6-TG exposure (Fig. 2B). We observed the previously reported (20, 21) phenomenon of “damage tolerance” of misincorporated bases in HCT116 cells following growth in the presence of up to 10 μM 6-TG, whereby cells which do not detect base damage are resistant to that damage. Interestingly, at 30 μM 6-TG, HCT116 cells were killed. In contrast, MLH1-expressing HCT116 3-6 cells demonstrated dramatically increased sensitivity to 6-TG. HCT116 3-6 cells were ~50-fold more sensitive to 6-TG (at 3–10 μM) than the genetically matched, MLH1-deficient HCT116 cells. Thus, the overall survival differences between HCT116 and HCT116 3-6 cells were reversed for IR compared to 6-TG.

Effect of MLH1 Deficiency on Cell Cycle Checkpoint Delays. We then examined differences in cell cycle checkpoint arrest between HCT116 and HCT116 3-6 cells after IR (Fig 3A). HCT116 3-6 cells demonstrated extensive G2-M cell cycle checkpoint arrest after 5 Gy, with cell populations in G2-M nearly three times that of parental HCT116 cells (Fig 3A). Interestingly, dramatic increases in G2-M arrest in HCT116 3-6 compared with HCT116 cells did not directly correlate with the modest survival differences (i.e., 46%; SER, 1.46) observed between these cell lines (Fig. 2). G0-G1 cell cycle checkpoint arrest was rather small in either cell line within the first 12 h after IR (see Figs. 3 and 4). In fact, although these cells express a damage-inducible wild-type p53 (shown below), both cell lines failed to demonstrate an immediate G0-G1 cell cycle arrest within the first 12 h after irradiation. Significant G1 arrest (increasing 15–20%) was
Fig. 3. Cell cycle regulation in MMR-deficient parental HCT116 and corrected HCT116 3-6 cells after IR or 6-TG. Shown are representative flow cytometry DNA histograms of HCT116 (hMLH1⁺) and HCT116 3-6 (hMLH1⁻) cells treated with IR (A) or 6-TG (B). Cells were seeded onto 10-cm² plates as described in “Materials and Methods” and treated 24 h later with either 5 Gy (A) or continuously exposed to 0.625 µg/ml (3.0 J/m²) 6-TG (B). Cells (Y-axis) were analyzed for relative DNA content (X-axis) by flow cytometric analyses at various times as described in “Materials and Methods.” Upper right-hand numbers, percentage of cells in G1-M. Experiments were performed at least three times, each in duplicate.

only observed at times >12 h after irradiation for HCT116 cells and at >24 h for HCT116 3-6 cells (Fig. 4A). The lack of a G1 arrest at early times after irradiation was apparently due to the more dramatic and earlier G2-M arrest observed in these cells after IR (Fig. 4C). More importantly, we noted that the G2-M arrest observed in irradiated HCT116 3-6 cells lasted 12–24 h longer than similarly irradiated HCT116 cells (Figs. 3A and 4). For example, at 24 h after 5 Gy, ~70% of irradiated HCT116 3-6 cells were in G2-M compared with only ~30% of irradiated HCT-116 cells, equivalent to those found in unirradiated HCT116 cells (Fig. 4C); these data demonstrate that by 24 h, HCT116 cells were no longer delayed in G2-M. The enhanced G2-M arrest observed in HCT116 3-6 cells was dose dependent up to 8 Gy, when a significant portion of cells was arrested (>85%) in this phase of the cell cycle (Fig. 5A). In contrast, irradiated HCT116 and HCT116 3-6 cells demonstrated no statistically significant difference in progression into the S-phase of the cell cycle (Fig. 4B), where dramatic decreases in S phase were observed between 2 and 12 h after irradiation in both cell lines, with very little recovery at later times. The persistent depletion of the S-phase population from 12 h after irradiation in both HCT116 and HCT116 3-6 cells after IR treatment
The enhanced G2-M cell cycle arrest, without functional differences in G1-S-phase cell cycle transition observed in irradiated HCT116 cells, was more directly demonstrated by the addition of nocodazole, an agent that causes cells to arrest at the G2-M phase of the cell cycle. We reasoned that if the function of the MLH1 protein was to regulate G1 cell cycle arrest or promote G1-S transition after IR, the exodus from G1 (and/or transition into S-phase) would be greater in HCT116 compared with HCT116 cells. We treated cells with IR and immediately exposed each cell line to 40 nM nocodazole to set up a permanent G2-M cell cycle arrest that prevented cells from recycling back into G1. The results demonstrate that HCT116 and HCT116 3-6 cells were identical in their progression out of G1 (Fig. 6A), their emergence into S phase (Fig. 6B), and their arrest in G2-M (Fig. 6C) after IR, due to the inhibition of microtubule formation caused by 40 nM nocodazole. These data strongly suggest that differences observed between HCT116 and HCT116 3-6 cells in G0-G1 cell populations following IR (Fig. 4) were, in fact, due to the lack of a G2-M cell cycle block in HCT116 cells after IR, resulting in a subsequent recycling of cells from G2-M back into G0-G1. The addition of 40 nM nocodazole corrected the G2-M cell cycle responses of HCT116 cells, making them similar to HCT116 3-6 cells after IR.

6-TG Responses. Previously, a 50-fold enhancement of survival of HCT116 over HCT116 3-6 cells was reported by Hawn et al. (20) and Koi et al. (21) after MNNG or 6-TG exposures. Furthermore, it was shown that HCT116 3-6 cells responded to these agents by arresting in G2-M, whereas HCT116 cells continued to cycle normally, demonstrating damage tolerance. Using analogous conditions (20, 21), we were able to demonstrate a similar difference in cytotoxicity (Fig. 2B) and cell cycle distribution (Fig. 3B) after 6-TG treatment in HCT116 3-6 compared with HCT116 cells. As noted (20, 21), parental HCT116 cells demonstrated little or no change in the percentage of cells in G2-M after 6-TG exposure. Accumulation of HCT116 3-6 cells in the G2-M phase of the cell cycle after 6-TG treatment was dose dependent; however, a similar relationship between 6-TG dose and G2-M cell cycle arrest for HCT116 cells was not observed (Fig. 5B).
Fig. 6. Effect of nocodazole-induced G2-M arrest on the cell cycle distribution of HCT116 or HCT116 3-6 cells after IR. Log-phase HCT116 or HCT116 3-6 cells were irradiated and immediately treated with or without 40 nM nocodazole to block cells at G2-M and prevent reentry into G0-G1. Note that nocodazole-treated, irradiated HCT116 (MLH1+; closed symbols) and HCT116 3-6 (MLH1−; open symbols) cells transited G1 (A) and S phase (B) equally but more slowly than those treated with nocodazole alone, presumably due to a modest G1 arrest induced by IR in both cell lines. Both cell lines also accumulated in G2-M (C) at the same rate with or without IR in the presence of 40 nM nocodazole. Thus, both cell lines appeared identical in all phases of cell cycle progression after IR in the presence of 40 nM nocodazole. Conditions were: circles, nocodazole alone; squares, nocodazole + 5 Gy; triangles, untreated control cells. The data for cells treated with 5 Gy alone (data not shown) are similar to those reported in Fig. 4.

Effect of MLH1 Deficiency on Damage-Inducible p53 Responses. Although parental and corrected HCT116 cells exhibited differences in radiation sensitivity and cell cycle alterations, both cell lines demonstrated similar immediate IR-induced nuclear (data not shown) and whole-cell p53 protein responses (Fig. 7, A and B; see similar p53 induction responses at each 4-h time point for each cell line at all doses of IR). However, the kinetics of p53 induction differed significantly between the two genetically matched cell lines. Induction of nuclear p53 protein levels in MMR-deficient HCT116 cells were more transient. Initial high levels of p53 in HCT116 cells decreased by 12–24 h in a dose-dependent manner (Fig. 7A). In contrast, elevated levels of p53 protein (~7–10-fold higher than in nonirradiated cells) were long lived (lasting greater than 48 h; data not shown) in HCT116 3-6 cells (Fig. 7B), although the cell cycle distribution of these irradiated cells varied widely over the course of the experiment (Fig. 3A). Similar p53 protein levels were also observed in confluence-arrested HCT116 or HCT116 3-6 cells (data not shown).

No correlation between p53 induction (Fig. 7) and the timing or overall extent of G2-M cell cycle checkpoint arrest (Figs. 3A, 4, and 5) were noted.

We also noted a dramatic induction of both nuclear and whole-cell p53 steady-state protein levels in HCT116 3-6 cells after 6-TG exposure, whereas similar increases were not observed in HCT116 cells, even after 6 days of continuous drug exposures of up to 3 μM 6-TG (Fig. 7, C and D). Both nuclear (data not shown) and whole-cell p53 protein level increases were, however, noted at a dose of 6 μM 6-TG (Fig. 7C). The absence of a p53 induction response in HCT116 (hMLH1−) cells after 6-TG exposure suggests a role of the MLH1 protein in both the detection of mismatch DNA base damage created by 6-TG and possibly in the intracellular signaling events [via MLH1 (MMR)-mediated induction of SSBs and/or DSBs], which elicited the damage-inducible p53 posttranscriptional process (37, 50). We also noted that even in the presence of a strong p53 response in HCT116 cells after a dose of 6 μM 6-TG (Fig. 7C), no G2-M cell cycle arrest was observed (Figs. 5B). These data suggest that p53 responses were not involved in MLH1-mediated G2-M cell cycle arrest.

Loss of Functional p53 Responses Do Not Affect MLH1-mediated G2-M Cell Cycle Checkpoint Arrest. Another interpretation of the data in Figs. 2–7 could be that the MLH1 protein functions in conjunction with nuclear p53 protein induction responses to regulate G2-M cell cycle arrest or in G1→S transition after IR. To investigate this, we transfected HCT116 and HCT116 3-6 cells with a retroviral vector that constitutively expressed the papillomavirus E6 protein as described in “Materials and Methods.” The E6 protein binds and targets p53 for ubiquitination and proteolysis, and results in loss of G1 cell cycle checkpoint arrest induced by DNA-damaging agents (51–53). The efficacy of E6 in targeting and removing available p53 protein in HCT116 and HCT116 3-6 cells, before and after 5 Gy, is demonstrated in Fig. 8. E6 expression did not affect MLH1 protein levels in HCT116 3-6 cells. Expression of E6 in transfected cells was also confirmed by PCR (data not shown). Transfected and nontransfected HCT116 and HCT116 3-6 cells were then examined for cell cycle distribution effects and for p53, MLH1, and MSH2 steady-state protein level changes after IR or 6-TG.

The loss of p53 expression and induction responses in HCT116 or HCT116 3-6 cells after IR caused by E6 transfection (Fig. 8) did not affect the overall cell cycle redistribution kinetics of irradiated HCT116 or HCT116 3-6 cells (Fig. 9), as observed previously for nontransfected cells (Figs. 3A and 4). Similar differences in G2-M cell populations between E6-transfected compared with nontransfected HCT116 and HCT116 3-6 cells were apparent 12–24 h after IR (compare data in Fig. 9C with that in Fig. 4C). Cells expressing the MLH1 protein (HCT116 3-6) demonstrated more dramatic and long-lasting G2-M cell cycle checkpoint delays compared with cells not expressing the protein (HCT116), regardless of E6 protein expression. We also noted that E6-expressing HCT116 or HCT116 3-6 cells were not different in their progression from G1, regardless of E6 protein expression.

No correlation between p53 induction (Fig. 7) and the timing or overall extent of G2-M cell cycle checkpoint arrest (Figs. 3A, 4, and 5) were noted.
ROLE OF MLH1 IN G2-M ARREST

Fig. 7. p53 steady-state induction responses in HCT116 and HCT116 3-6 cells after IR or 6-TG. Log-phase cells were treated with IR or 6-TG, and whole-cell extracts were prepared at the indicated times as described in “Materials and Methods.” Extracts were loaded and separated by 6% SDS-PAGE and analyzed by Western immunoblotting as described in “Materials and Methods.” Representative dose-response and time course p53 protein expression responses after 2, 5, or 8 Gy IR for HCT116 (A) or HCT116 3-6 (B) cells are shown; similar responses were noted in nuclear preparations (data not shown). A dose-response and time course steady-state protein expression pattern for MLH1 (top blot, M, 84,000) and p53 (middle blot) in HCT116 cells (C) or HCT116 3-6 cells (D) after 1.5, 3, or 6 µM 6-TG is also demonstrated. The lower band in the MLH1 blots (M, ~70,000 polypeptide) was nonspecific and served as an internal loading control for HCT116 cells. Blots were representative of results from three or more separate experiments and Western analyses.

data in Fig. 9C with Fig. 4C), strongly suggesting that neither p53 nor G1 cell cycle arrest influenced the MLH1-mediated G2 arrest signals within the cell.

MMR Protein Levels Were Not Affected by p53 Expression. We also found that the steady-state levels of MLH1 or MSH2 proteins were not affected by the loss of p53, nor were the levels of these two proteins altered in irradiated HCT116 E6 or HCT116 3-6 E6 cells (Fig. 8), similar to the responses found with nontransfected cells (Fig. 1). E6 expression in HCT116 or HCT116 3-6 cells also did not affect survival differences after IR, and radiation survival curves overlapped those presented in Fig. 2A (data not shown). Taken together, these data strongly suggest that p53 does not play a role in G2-M cell cycle checkpoint arrest or in survival differences observed between cells that express MLH1 (HCT116 3-6) or in cells that have lost expression of this MMR protein (e.g., in HCT116 cells).

Primary Embryonic Fibroblasts from Transgenic MLH1 Knockout Mice Also Demonstrated Deficient G2-M Cell Cycle Checkpoint Responses. We were concerned that the apparent MLH1-dependent, G2-M cell cycle checkpoint arrest characterized above for HCT116 3-6 compared with HCT116 cells was cell-type specific. Therefore, we performed identical cell cycle regulatory studies using primary embryonic fibroblasts derived from MLH1 knockout siblings. Primary embryonic fibroblasts were isolated as described in “Materials and Methods” from wild-type or MLH1 knockout fetuses, and their responses to IR and 6-TG were compared. In control experiments, we examined MLH1 and MSH2 protein expression in three representative populations: one homozygous wild-type at the MLH1+/+ locus, one heterozygous for MLH1+/−, and one homozygous mutant at the MLH1−/− locus (Fig. 10). As expected, heterozygous and homozygous wild-type cells expressed normal basal steady-state protein levels of both MLH1 and MSH2 (Fig. 10), at levels similar to those found previously in IMR-90 (48) or HCT116 (Fig. 1) cells. These data suggest that the presence of one MLH1 gene was apparently sufficient for normal steady-state protein levels as measured by Western blot analyses. As expected, homozygous mutant MLH1−/− cells did not express the murine MLH1 protein but did express normal levels of MSH2. As with HCT116 or HCT116 3-6 cells, the steady-state protein levels of MSH2 or MLH1 were unaltered after IR (Fig. 10A) or 6-TG (Fig. 10B) treatments.

We then used these genotypically matched, embryonic fibroblasts for cell cycle distribution experiments after 6-TG or IR, as performed with the HCT116 human colon cancer cell system. As with HCT116 (hMLH1−/−) cells, primary embryonic fibroblasts from MLH1 knockout mice failed to arrest at the G2-M cell cycle checkpoint at 24 h after IR (Fig. 11A) or at 6 days after 6-TG exposure (Fig. 11B). In contrast, wild-type embryonic fibroblasts, which expressed normal levels of the MLH1 protein, demonstrated a dramatic G2-M cell cycle checkpoint arrest in response to increasing doses of IR (Fig. 11A) or 6-TG (Fig. 11B). Following 12 Gy, MLH1+/− embryonic fibroblasts demonstrated 2-fold more cells in G2-M than MLH1−/− embryonic fibroblasts. The G2-M cell cycle responses in embryonic fibroblasts after
ently by associating with the meiotic chiasma (19). On a broader level, cells arrested to a significantly greater extent than heterozygous MLH1+/− cells, and homozygous-deleted MLH1−/− cells demonstrated very little G2-M cell cycle arrest, even after 12 Gy. This gene-dosage effect was interesting in light of the apparent equivalent MLH1 protein levels found in EF37 (MLH1+/+) compared with EF19 (MLH1−/−) cells (Fig. 10). Further detailed analyses of cell cycle redistribution kinetics over a 72-h time period indicated that IR-treated wild-type or MLH1 knockout murine embryonic fibroblasts demonstrated no apparent differences in G0-G1 or progression into S phase, except for the immediate G2-M cell cycle arrest responses noted in Fig. 11. These data using murine primary embryonic fibroblasts further confirm a role for the MLH1 protein in G2-M cell cycle checkpoint arrest and demonstrate that the HCT116 model cell system is functionally similar to cells derived from wild-type or MLH1 knockout mice after IR or 6-TG.

**DISCUSSION**

Although DNA MMR proteins are important for detection, removal, and repair of mismatched bases in DNA, other functions have been proposed. The MLH1 protein plays an important role in postreplication MMR and was previously suggested to be important in G2-M cell cycle arrest after 6-TG exposure (12, 20, 21). Other data indicated a role for the MLH1 protein in meiotic segregation, apparently by associating with the meiotic chiasma (19). On a broader level, the MMR genes (i.e., MLH1, MSH2, and PMS2) were also implicated in transcription-coupled repair of environmentally induced DNA lesions (54, 55), indicating a role for these proteins in cellular responses to DNA damage. Details of the involvement of the MLH1 protein in these processes are, however, poorly understood. We studied the cellular responses of genetically matched MMR-proficient compared with MMR-deficient cells after DNA-damaging agents (such as IR and 6-TG) to better understand how DNA MMR was coupled to stress responses, including DNA lesion recognition and processing, cell cycle checkpoint arrests, and changes in gene expression. We also explored the potential role of MLH1 in G2-M cell cycle checkpoint regulation, which may be essential for survival after IR (33). Given the poor understanding of processes that regulate the G2-M cell cycle checkpoint after IR, we wanted to determine the importance of MLH1 in G2-M and eliminate potential roles for this protein in G1 phase transition or in the G1 cell cycle checkpoint delays.

We demonstrated that cells lacking MLH1 have both different and overlapping responses to IR or 6-TG, presumably due to differences in DNA lesions created by these very different DNA-damaging the poor understanding of processes that regulate the G2-M cell cycle checkpoint after IR, we wanted to determine the importance of MLH1 in G2-M and eliminate potential roles for this protein in G1 phase transition or in the G1 cell cycle checkpoint delays.

We demonstrated that cells lacking MLH1 have both different and overlapping responses to IR or 6-TG, presumably due to differences in DNA lesions created by these very different DNA-damaging
agents. IR directly causes SSBs and DSBs, which activate immediate damage-recognition responses. Additionally, DNA breaks may persist due to subsequent processing of DNA lesions by DNA base and nucleotide excision repair. DSBs are considered to be the most important lethal lesions created by IR (31, 34, 56) and have been linked to apoptosis (57). We demonstrated that the p53 response of both MMR-proficient and MMR-deficient cells was initially equal after DNA damage created by IR, presumably because X-irradiation induces a rapid G2-M arrest in both cell lines within 12 h (Figs. 3A and 4), resulting in a G2-M arrest; (b) MLH1 (and functional MMR) acts to process DNA damage created by IR before and after DNA strand break repair, and this proofreading capacity of MMR (perhaps by causing additional DNA breaks) results in a longer G2-M arrest than observed in MLH1-deficient cells; or (c) the MLH1 protein serves a coupling, dual role to initiate proofreading repair of mismatched lesions after DNA break repair and also directly interacts with the cell cycle machinery to regulate G2-M. The prolonged induction of p53 in MMR-proficient HCT116 3-6 cells was independent of cell cycle alterations and was quite long-lived, lasting beyond 24 h after IR (Fig. 7B). Similar p53 responses have been shown to be related to the presence of DNA strand breaks (37), suggesting that MMR function leads to additional breaks after IR. In contrast, the p53 response in HCT116 (MMR−) cells was more transient after IR (Fig. 7A). A similar relationship between MLH1 expression and G2-M cell cycle arrest after IR was also found in mouse cells lacking or expressing MLH1. These cellular responses may be consistent with a MMR-
mediated, proofreading creation of DSBs, which then causes the otherwise unrelated responses of p53 induction and G2-M arrest. The creation of DSBs by bacterial MMR has been observed in vitro using unmethylated plasmid DNA. A low level of DSBs was observed at unmethylated GATC sites (58). An alternative hypothesis [also offered by Kat et al. (59)] entails futile cycling as the cell attempts to remove mispaired or damaged bases, possibly resulting in DSBs when a replication fork encounters such repair complexes. Further investigations will be required to determine whether MMR processing of 6-TG or IR DNA lesions results in DSBs or SSBs.

Our data are also equally consistent with a direct role for the hMLH1 protein in regulating G2-M arrest. It was recently demonstrated that the MLH1 protein interacts with proliferating cell nuclear antigen in yeast (60, 61), suggesting that this protein interacts with cell cycle regulatory proteins. Data presented in this report strongly suggest that expression of MLH1 regulates G2-M after IR. The MLH1 protein has no known enzymatic function and no functional motifs that would explain a DNA lesion-forming function; it does not directly interact with DNA lesions, and it is not involved in direct DNA repair processing of altered bases (4, 62). We are presently examining DNA lesion formation in MMR-proficient compared with MMR-deficient cells after equitoxic doses of IR as a first step in differentiating between these three possible hypothetical roles for MLH1 in G2-M cell cycle regulation.

It was interesting that HCT116 and HCT116 3-6 cells were not dramatically different in terms of radiation sensitivity (Fig. 2), despite the much more dramatic G2-M cell cycle responses noted in MMR-proficient compared with MMR-deficient cells. Unfortunately, survival (i.e., colony-forming assays) for genetically matched murine embryonic fibroblasts could not be accurately measured due to their inability to form colonies. However, in terms of changes in cell numbers over time, murine fibroblasts from MLH1 knockout or wild-type embryos demonstrated approximately equal sensitivity to ionizing radiation (data not shown). Recent studies with established murine fibroblast cell lines from MLH1-, PMS2-, and MSH2-deficient mice also demonstrated little differences in survival responses after IR compared with cell lines from wild-type littermates (63). In both genetically matched cell systems tested in this study, the deficiency in MLH1 expression led to dramatically reduced G2-M responses with only slight survival differences after IR (as shown in Fig. 2). This strongly suggests that the G2-M checkpoint is one factor in determining overall survival following IR but is clearly not the only factor. Other immediate and long-term genetic responses could account for the lack of a more dramatic cell death response between these MLH1-expressing compared with MLH1-deficient cells. The net result of MMR proficiency would be a slightly enhanced survival of accurately repaired cells and elimination of genetically altered cells compared with MLH1-deficient cells after IR.

In contrast to responses observed with IR, only cells that expressed a functional MMR system (e.g., HCT116 3-6, wild-type, or heterozygous embryonic fibroblasts) detected 6-TG after its incorporation into DNA. Cells lacking MMR function (i.e., HCT116 or embryonic fibroblasts from MLH1 knockout mice) demonstrated DNA damage tolerance to 6-TG. The cellular response to 6-TG depended exclusively on the presence of functional MMR processes. We hypothesize that cells with functional MMR recognized and removed 6-TG-sensitized DNA lesions, resulting in the formation of DNA breaks. This DNA damage (presumably DSBs), in turn, resulted in a subsequent p53 induction response (Fig. 7, C and D), G2-M cell cycle delay (Figs. 3B and 5B), and loss of survival, presumably due to DNA lesions created after MMR-mediated detection and processing. Although there is a possible direct regulatory role for the MLH1 protein in G2-M cell cycle checkpoint control, such responses may be an indirect result of the formation of DNA DSBs that are known to initiate arrest at this phase of the cell cycle (64). The results presented here suggest that DSBs are absent in MMR-deficient cells exposed to 6-TG, presumably because the MMR protein complex(es) that are required to produce DNA breaks at the sites of 6-TG incorporation are deficient or defective. This implies that the MLH1 protein is required for DNA lesion processing with subsequent formation of DNA breaks. However, it is possible that the MLH1 protein could also regulate the G2-M cell cycle checkpoint. Experiments directed at characterizing DNA lesion formation and repair after 6-TG or IR may resolve the potential role of the MLH1 protein in lesion processing, G2-M cell cycle regulation, or a dual function for this protein.

Although neither of the HCT116 cell lines demonstrate a significant G2-M arrest immediately after IR, our data indicate that MLH1 protein expression did not play a role in G1→S transition. Cells lacking MLH1 protein expression did not emerge from the G2 phase of the cell cycle or transit S-phase differently than cells that expressed this MMR protein (Fig. 4). Administration of nocodazole to irradiated MLH1-deficient HCT116 cells essentially corrected the observed cell cycle-arrest deficiency, making them look identical to damaged HCT116 3-6 cells after IR (Fig. 6). Finally, retrovirally mediated expression of E6 did not affect the differences in cell cycle regulation or survival observed between MMR-proficient and -deficient cells. Because E6 expression effectively eliminated the p53 protein (Fig. 8) and any delayed G1 checkpoint responses (Fig. 9) but had no effect on G2-M and survival differences between these MMR-proficient or -deficient cells, these data strongly suggest that neither p53 nor G1 cell cycle checkpoint responses were involved. It is interesting that HCT116 and HCT116 3-6 cells (both of which are proficient in p53 induction) arrested in G1 only after an earlier and more dramatic G2-M arrest.

Finally, our data indicate that MLH1 and MSH2 proteins are not damage inducible after either 6-TG or IR, and their expression levels were not altered in the G2-M phase of the cell cycle, nor were the levels of MSH2 altered by the presence of MLH1 (Figs. 1 and 10). Such constitutive expression throughout the cell cycle and lack of damage inducibility of MMR proteins are consistent with responses observed in yeast and bacteria. These data are consistent with our recent observations that the MLH1 and MSH2 proteins are expressed in all phases of the cell cycle (48). The above observations were not artifacts of the HCT116 system, because primary embryonic fibroblasts from MLH1 knockout mice behaved as HCT116 cells, showing an absence of a G2-M cell cycle arrest and a reduced and shortened G2-M arrest after 6-TG or IR exposures compared with their genetically matched wild-type cells.

The role of MMR deficiencies in the generation of neoplastic cells is of great interest. Knockout mice have been constructed that lack various MMR components. Interestingly, MSH2 (13, 65), PMS2 (66), and MLH1-deficient mice (19, 67) appear susceptible to lymphoid tumors early and throughout their lives. In MSH2 homozygous-deficient mice, intestinal and skin tumors appeared late and were generally not the cause of death (68). These results are in contrast to human kindreds susceptible to hereditary nonpolyposis colorectal cancer (2–6). Such kindreds carry one mutated allele of an MMR gene at birth and require a second mutagenic event to alter the remaining allele in tumors. These individuals rarely present with hematopoietic tumors. The fact that homozygous MMR-deficient mice develop normally and only demonstrate intestinal tumors late in life suggest that MMR-related tumorigenesis requires secondary events. The data presented in this report support the idea that a specific, environmentally induced stress may be necessary before neoplasia is initiated. Understanding how matched MMR-proficient and MMR-deficient cells cope with various DNA stresses should help elucidate factors...
involved in G2-M cell cycle checkpoints, as well as stress responses
that result in carcinogenesis.

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Defective Expression of the DNA Mismatch Repair Protein, MLH1, Alters G2-M Cell Cycle Checkpoint Arrest following Ionizing Radiation

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