Role of the hMLH1 DNA Mismatch Repair Protein in Fluoropyrimidine-mediated Cell Death and Cell Cycle Responses

Mark Meyers, Mark W. Wagner, Hwa-Shin Hwang, Timothy J. Kinsella, and David A. Boothman

Department of Radiation Oncology and the Ireland Cancer Center, Laboratory of Molecular Stress Responses, Case Western Reserve University, Cleveland, Ohio 44106-4942

ABSTRACT

DNA mismatch repair (MMR) is an efficient system for the detection and repair of mismatched and unpaired bases in DNA. Deficiencies in MMR are commonly found in both hereditary and sporadic colorectal cancers, as well as in cancers of other tissues. Because fluorinated thymidine analogues (which through their actions might generate lesions recognizable by MMR) are widely used in the treatment of colorectal cancer, we investigated the role of MMR in cellular responses to 5-fluorouracil and 5-fluoro-2'-deoxyuridine (FdUrd). Human MLH1+ and MMR-deficient HCT116 colon cancer cells were 18-fold more resistant to 7.5 μM 5-fluorouracil (continuous treatment) and 17-fold more resistant to 7.5 μM FdUrd in clonogenic survival assays compared with genetically matched, MLH1+ and MMR-proficient HCT116 3–6 cells. Likewise, murine MLH1+ and MMR-deficient CT-5 cells were 3-fold more resistant to a 2-h pulse of 10 μM FdUrd than their MLH1+ and MMR-proficient ME-10 counterparts. Decreased cytotoxicity in MMR-deficient cells after treatment with various methylating agents and other base analogues has been well reported and is believed to reflect a tolerance to DNA damage. Synchronized HCT116 3–6 cells treated with a low dose of FdUrd had a 2-fold greater G2 cell cycle arrest compared with MMR-deficient HCT116 cells, and asynchronous ME-10 cells demonstrated a 4-fold greater G2 arrest after FdUrd treatment compared with CT-5 cells. Enhanced G2 arrest in MMR-proficient cells in response to other agents has been reported and is believed to allow time for DNA repair. G2 cell cycle arrest as determined by propidium iodide staining was not a result of mitotic arrest, but rather a true G2 arrest, as indicated by elevated cyclin B1 levels and a lack of staining with mitotic protein monoclonal antibody 2. Additionally, p53 and GADD45 levels were induced in FdUrd-treated HCT116 3–6 cells. DNA double-strand break (DSB) formation was 2-fold higher in MMR-proficient HCT116 3–6 cells after FdUrd treatment, as determined by pulsed-field gel electrophoresis. The formation of DSBs was not the result of enhanced apoptosis in MMR-proficient cells. FdUrd-mediated cytotoxicity was caused by DNA-directed and not RNA-directed effects, because administration of excess thymidine (and not uridine) prevented cytotoxicity, cell cycle arrest, and DSB formation. hMLH1-dependent responses to fluoropyrimidine treatment, which may involve the action of p53 and the formation of DSBS, clearly have clinical relevance for the use of this class of drugs in the treatment of tumors with MMR deficiencies.

INTRODUCTION

Colorectal cancers are the third most common cancers in men and women. Deficiencies in genes of the MMR pathway have been found in hereditary nonpolyposis colorectal cancer as well as in sporadic cancers of the colon and other tissues. MMR is a vital mutation avoidance system that recognizes and repairs mismatched and unpaired bases in DNA that arise from DNA replication errors, 5-methylcytosine deamination, the action of chemical mutagens, and other processes (2–4). Interestingly, cells deficient in MMR are resistant to many DNA-damaging agents, including the base analogue 6-TG, the alkylating agent MNNG, and the adduct-forming drug cisplatin (5–8). It is postulated that these agents cause DNA lesions (through adduct formation or incorporation) that, by themselves, are not lethal. However, MMR attempts to process these modified bp as though they were replication errors but cannot complete the repair because one of the bases is abnormal. Thus, these persistent intermediates may serve as the important DNA lesions. Attempts at repair are then futile and often result in cell cycle arrest and perhaps apoptosis (9). MMR-deficient cells survive these treatments but consequently accumulate mutations (10–12).

The FP antimetabolite, 5-FU and its deoxyribonucleoside derivative, FdUrd, are the most widely used drugs in the treatment of advanced colorectal cancer (13–15). Whereas 5-FU is used for standard systemic chemotherapy, FdUrd, which generally has stronger antitumor activity but is more expensive and produces more systemic toxicity, is restricted to regional hepatic artery infusion or infusions using chronotherapy (15). The metabolites of 5-FU and FdUrd can cause both RNA- and DNA-directed cytotoxicities (16). RNA-directed effects are the result of incorporation of 5-fluoroUTP into RNA and subsequent alterations in RNA processing that can be rescued by Urd administration (17, 18). DNA-directed effects are attributable to the incorporation of FdUTP into DNA, or to the inhibition of TS by the formation of a ternary complex consisting of TS, FdUrd, and CH2-THF. Because TS is the central enzyme of de novo pyrimidine synthesis, its inhibition results in decreased intracellular dThd pools. This leads to imbalances in the dNTP pools as well as the inhibition of DNA synthesis (19). DNA-directed cytotoxicity can be reversed by rescue with excess dThd (20).

DNA-directed effects of the FPs may be more important for chemotherapy, whereas RNA-directed effects may be the cause of nonspecific cytotoxicity (15). For example, Urd was able to reverse 5-FU-induced myelosuppression and gastrointestinal toxicity without tumor protection (21, 22). Another study showed that gastrointestinal toxicity was attributable to RNA incorporation and not increasing dUMP levels (23). Whereas 5-FU is much more widely used in the clinic, FdUrd exerts its effects more predominantly on DNA than 5-FU does; FdUrd requires only phosphorylation by dThd kinase to form FdUMP, whereas 5-FU must first be converted to FdUrd after the addition of deoxyribose by dThd phosphorylase (24). Therefore, many clinical protocols have the goal of making 5-FU a more DNA-directed drug by enhancing the inhibition of TS. For example, it is standard clinical practice to administer 5-FU with leucovorin, a precursor of...
CH₂-THF; this treatment leads to stabilization of the ternary complex and hence a more efficient inhibition of TS (25).

The role of MMR in drug cytotoxicity has been explored using various genetically matched cells containing altered MMR proficiency. One commonly used system uses the hMLH1-deficient HCT116 human colon cancer cell line and a corrected clone (HCT116 3-6), in which a normal human chromosome 3 (which contains the hMLH1 gene) was introduced by microcell fusion (6). Restoration of MMR led to increased sensitivity to MNNG and 6-TG. HCT116 3-6 cells also exhibited a more pronounced p53-independent G ÷ M arrest compared with parental HCT116 cells after treatment with 6-TG or ionizing radiation (5, 26). Another newly developed system uses spontaneously immortalized embryonic fibroblasts from the MLH1-/- mouse that were transfected with hMLH1 cDNA (ME-10) or empty vector (CT-5). Introduction of hMLH1 reduced the mutation rate and restored G ÷ M- arrest responses in ME-10 cells (27).

We examined the responses of MMR-proficient and -deficient cells to 5-FU or FdUrd. Our data indicate that hMLH1-meditated, MMR processes play a role in G ÷ M cell cycle checkpoint arrest responses and cytotoxicity after 5-FU or FdUrd exposures. Loss of MMR leads to damage tolerance to the DNA-directed effects of FPs.

MATERIALS AND METHODS

Cells, Culture Conditions, and Reagents. HCT116 and HCT116 3-6 cell lines were generously provided by Dr. C. Richard Boland (University of California, San Diego, CA). A normal human chromosome 3, which contains the hMLH1 gene, was introduced by microcell fusion into HCT116, an hMLH1-deficient human colon cancer cell line. The resulting MMR-proficient cell line was named HCT116 3-6 (6). CT-5 and ME-10 clones were obtained from Drs. Andrew Buermeyer and R. Michael Liskay (Oregon Health Sciences University, Portland, OR) in which spontaneously immortalized embryonic fibroblasts from the MLH1-/- mouse were transfected with hMLH1 cDNA (ME-10) or empty vector (CT-5; Ref. 27). All cells were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 10% FBS (HyClone, Logan, UT) and were free of Mycoplasma contamination. 5-FU, FdUrd, dThd, and Urd were obtained from Sigma Chemical Co. (St. Louis, MO). Each was dissolved in water (FdUrd, dThd, and Urd) or DMSO (5-FU) and stored at −20°C for no longer than 3 months. Colcemid dissolved in HBSS was obtained from Life Technologies, Inc.

Clonogenic Survival Analyses. Survival was assessed by colony-forming ability using standard techniques (5). Only colonies of >50 normal-appearing cells were counted. Human cells were treated continuously with 5-FU or FdUrd for 10 days, with fresh medium containing drug added every 3 days. For rescue experiments, a 10-fold molar excess of dThd or a 40-fold molar excess of Urd was added simultaneously with FdUrd. Mouse cells were treated for 2 h with 10 μM FdUrd, then cultured an additional 7 days. For rescue experiments, 10 μM dThd or Urd was added to the culture medium after FdUrd treatment was completed. The statistical significance of the results was determined by a Student t test.

Cell Cycle Analyses. Human cells were first synchronized by allowing them to grow to confluence and then maintained for 2 days in DMEM with 0.1% FBS to enhance G1-G1 arrest. To induce proliferation, the confluent cell monolayers were then dissociated (using 0.05% trypsin with 0.53 mM EDTA), and cells were replated at 1:4–1:10 dilutions in DMEM containing 10% FBS. FdUrd was then added 16 h after release from confluence; this corresponds to the time the majority of cells begin S-phase. Synchronization allowed us to isolate the DNA-directed effects of FdUrd (because the drug was added immediately before the time it could be incorporated into DNA during replication and/or inhibit TS activity), as well as bypass p53-mediated G1 checkpoints. If indicated, a 10-fold molar excess of dThd was added simultaneously with the drug. Asynchronous mouse cells were treated for 24 h with various concentrations of FdUrd, then the medium containing drug was removed and replaced with fresh medium. At the indicated time points, cells were dissociated, fixed, stained with propidium iodide, and analyzed on a Coulter Epics XL as described (28). Cell cycle populations were analyzed using ModFit LT version 2.0 software (Verity Software House, Topsham, ME).

Cells treated as above were stained with the MPM-2, fluorescein-5-EX succinimidyl ester IgG1-conjugated mouse monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) by fixing them in an ice-cold solution containing 10% PBS and 90% ethanol, washing twice in PBS containing 1% FBS, incubating with 0.25 μg of MPM-2 antibody per 106 cells for 30 min at 37°C, chilling cells for 10 min at 4°C, washing twice with PBS containing 1% FBS at 4°C, and finally staining cells in a solution containing 1 mg/ml RNase A, 50 μg/ml propidium iodide, and 0.1% NP40 for 30 min at 37°C. Cells treated continuously with 200 ng/ml Colcemid, an agent which causes a mitotic arrest, served as positive controls for MPM-2 staining. The expression of cyclin B1 (in synchronized human cells treated with 0.25 μM FdUrd) throughout the stages of the cell cycle was determined using flow cytometry as described (29, 30).

Preparation of Protein Extracts and Western Blot Analyses. Whole-cell extracts and Western blots were prepared as described (31). Human cells were synchronized and treated with 0.25 μM FdUrd in the same manner as described for cell cycle analyses. For Western blot analyses, primary antibodies against the following proteins were used at the indicated dilutions: hMLH1 (Ab-2; Oncogene Research Products, Boston, MA), 1:100; hMSH2 (Ab-1; Oncogene Research Products), 1:500; hPMS2 (Ab-1; Oncogene Research Products), 1:200; p53 (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA), 1:3000; GADD45 (C-4; Santa Cruz Biotechnology), 1:100; cyclin B1 (H-20; Santa Cruz Biotechnology), 1:500; and β-actin (AC-15; Sigma Chemical Co.), 1:20,000. Immunoprecipitations of p34cdc2 were performed as described (31), and blots made from these immunoprecipitates were immunostained with antibodies against phosphotyrosine (PY99; Santa Cruz Biotechnology; 1:2000) and p34cdc2 (C-19; Santa Cruz Biotechnology; 1:2000). Detection was done using the appropriate antiserum or antirabbit IgG horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and the SuperSignal chemiluminescence substrate system (Pierce, Rockford, IL) on Fuji RX medical X-ray film (Fuji Photo Film, Tokyo, Japan).

DSB and Apoptosis Measurements. DSBs induced by FdUrd treatment were quantitated by PFGE as described (32). Asynchronous HCT116 or HCT116 3–6 cells were treated with 0–10 μM FdUrd continuously for up to 72 h. Both attached and floating cells were collected. Gels were stained in ethidium bromide and quantitated using a FluorImager (Molecular Dynamics, Sunnyvale, CA). The levels of apoptosis were measured using the APO-BRDU flow cytometry kit (Phoenix Flow Systems, San Diego, CA) following the manufacturer’s instructions; this is a modified TUNEL assay using dual-color flow cytometry.

RESULTS

hMLH1 Deficiency Allows Resistance to 5-FU and FdUrd. Clonogenic assays were performed to determine the survival of HCT116 (MMR-deficient) compared with HCT116 3-6 (MMR-proficient) cells subsequent to continuous treatments with various doses of 5-FU or FdUrd (Fig. 1). HCT116 cells were 18-fold more resistant to 7.5 μM 5-FU (Fig. 1A) and 17-fold more resistant to 7.5 μM FdUrd (Fig. 1B) compared with genetically matched HCT116 3-6 cells. The survival difference between these two cell lines was more apparent at higher doses; for example, with 5-FU treatment, the LD90 for HCT116 cells was 6.9 μM and for HCT116 3-6 cells was 3.5 μM (a 2.0 ratio) and for FdUrd treatment, the LD90 for HCT116 cells was 4.8 μM and for HCT116 3-6 cells was 2.8 μM (a 1.7 ratio). Interestingly, a significant difference in survival between HCT116 and HCT116 3-6 cells was not observed after shorter exposures to 5-FU or FdUrd (24 h or less); this is in agreement with previous reports (33, 34). This resistance to 5-FU and FdUrd is consistent with the phenomenon of damage tolerance, whereby MMR-deficient cells are more resistant to agents such as MNNG and 6-TG (35, 36).

To determine whether this cytotoxicity occurred at the DNA or RNA level, FdUrd-treated cells were incubated simultaneously with a 10-fold molar excess of dThd or a 40-fold molar excess of Urd (relative to the FdUrd dose) to determine whether these agents could rescue FdUrd-induced cytotoxicity (Fig. 1B). dThd completely res-
with MMR-deficient CT-5 cells (Fig. 2). Subsequent incubation with 3-fold more sensitive to a 2-h exposure to 10 μM dThd was added, the addition of 10 μM dThd by itself had no effect on survival; the surviving fraction was within 1 ± 0.2 (SE). Data, mean ± SE. Comparisons between CT-5 and ME-10 cells were made using the paired Student t test. * P < 0.05.

ROLE OF hMLH1 IN RESPONSE TO FLUOROPYRIMIDINES

hMLH1 Restores G2-M Arrest after FdUrd Treatment. The cell cycle distributions of MMR-proficient and -deficient cells subsequent to exposure to FdUrd were examined (Fig. 3). hMLH1-deficient mouse cells caused by 5-FU or FdUrd treatment. HCT116 (○) or HCT116 3-6 (●) colon cancer cells were treated with various concentrations of 5-FU (A) or FdUrd (B) continuously for 10 days, and colony-forming ability was assessed. B, a 10-fold molar excess of dThd (relative to the FdUrd concentration) was added simultaneously with FdUrd (●, HCT116 + dThd; ■, HCT116 3-6 + dThd). The addition of dThd by itself had little effect on survival; at all doses, the surviving fraction was within 1.0 ± 0.3 (SE). Data, mean ± SE. In most cases, the symbols hide the error bars. Comparisons between HCT116 and HCT116 3-6 cells were made using the paired Student t test. * P < 0.05.

Fig. 1. Cytotoxicity of MMR-deficient (HCT116) or MMR-proficient (HCT116 3-6) human cells caused by 5-FU or FdUrd treatment. HCT116 (○) or HCT116 3-6 (●) colon cancer cells were treated with various concentrations of 5-FU (A) or FdUrd (B) continuously for 10 days, and colony-forming ability was assessed. B, a 10-fold molar excess of dThd (relative to the FdUrd concentration) was added simultaneously with FdUrd (●, HCT116 + dThd; ■, HCT116 3-6 + dThd). The addition of dThd by itself had little effect on survival; at all doses, the surviving fraction was within 1.0 ± 0.3 (SE). Data, mean ± SE. In most cases, the symbols hide the error bars. Comparisons between HCT116 and HCT116 3-6 cells were made using the paired Student t test. * P < 0.05.

Fig. 2. Cytotoxicity of MMR-deficient or MMR-proficient mouse cells caused by FdUrd treatment. Spontaneously immortalized MMR-deficient CT-5 (○) or MMR-proficient ME-10 (●) embryonic fibroblasts were treated for 2 h with 10 μM FdUrd and then assayed for clonogenic survival 7 days later. Subsequent to FdUrd treatment, 10 μM dThd was added. The addition of 10 μM dThd by itself had no effect on survival; the surviving fraction was within 1 ± 0.2 (SE). Data, mean ± SE. Comparisons between CT-5 and ME-10 cells were made using the paired Student t test. * P < 0.025.

CT-5 and ME-10 mouse fibroblasts were also examined for cell cycle changes during FdUrd treatments (Fig. 4). Asynchronous cells were treated for 24 h with various doses of FdUrd and then analyzed 24 h after drug removal. In agreement with the results observed using the HCT116 cell system, MMR-proficient ME-10 cells demonstrated a greatly enhanced G2-M arrest. This G2-M arrest lasted at least 48 h after treatment. Exposure times of 12 h or less to FdUrd (∼2.5 μM)
Staining with MPM-2 Indicates Arrest Is in G2, not M, Phase. The MPM-2 antibody recognizes a phosphorylated LTPLK or FTPLQ epitope in a class of proteins (such as microtubule-associated protein 2, DNA topoisomerase IIα, and CDC25) that are phosphorylated at the onset of mitosis by MPF, a complex consisting of the catalytic cyclin-dependent kinase p34CDC2, a regulatory cyclin B protein, and associated proteins (38–40). Thus, positive staining by MPM-2 is an accurate indicator of cells in mitosis. HCT116 and HCT116 3-6 cells were stained with MPM-2 and analyzed by flow cytometry to determine whether the G2-M cell cycle arrest (as indicated by propidium iodide staining) occurred primarily in the G2 or M phase of the cell cycle (Fig. 5, A and B). Synchronized cells that were untreated, continuously exposed to 200 ng/ml Colcemid (a drug which destabilizes microtubules and thus causes a mitotic arrest), or treated continuously with 0.25 μM FdUrd (as in Fig. 3) were examined. By comparison with Fig. 3, >50% of both HCT116 and HCT116 3-6 cells were in G2-M at 16 h after FdUrd addition as determined by propidium iodide staining, but <5% of these cells were MPM-2-positive (Fig. 5, A and B). Moreover, >50% of FdUrd-treated HCT116 3-6 cells remained in G2-M for at least 56 h (Fig. 3), yet there was still very little staining with MPM-2 (Fig. 5B). Clearly, FdUrd-treated G2-M cells were MPM2-negative, whereas flow cytometry indicated that a significant portion of the cells were arrested in G2-M. A pulse of synchronized, untreated mitotic cells was not apparent, because it would have occurred between 20 and 32 h (Fig. 5, A and B). These data strongly suggest that FdUrd treatment causes an MMR-dependent G2 arrest.

Cyclin B1 levels were then examined by flow cytometry. Entry into mitosis requires MPF activation, which depends on an increase in cyclin B1 expression and dephosphorylation of p34CDC2, whereas progression through mitosis requires MPF inactivation, which in part depends on cyclin B1 degradation (38, 41). Thus, cyclin B1 levels are generally higher in G2 cells than in mitotic cells. The observed increases in cyclin B1 expression in FdUrd-treated HCT116 3-6 cells relative to HCT116 cells further confirmed that a G2 arrest had occurred (Fig. 5C).

Cyclin B1, p53, and GADD45 Levels Increase following FdUrd Treatment in hMLH1-corrected HCT116 3-6 Cells. Western immunoblot analyses were performed then, using whole cell extracts from synchronized HCT116 and HCT116 3-6 cells treated with 0.25 μM FdUrd, to examine differences in molecular stress responses (Fig. 6A). hMLH1 was absent from HCT116 cells, and its steady-state level in HCT116 3-6 cells was not affected by FdUrd treatment. hPMS2, the pairing partner of hMLH1 (42), was detected in HCT116 3-6 cells, presumably because of its stabilization by association with hMLH1 (43). Interestingly, levels of both hMSH2 (another MMR protein; Ref. 44) and hPMS2 were enhanced in HCT116 3-6 cells at a time coinciding with G2 arrest responses. Minor changes in MMR protein levels attributable to cell cycle changes have been reported (28, 45). Levels of the DNA damage-inducible p53 protein (46) increased significantly shortly after FdUrd exposure in both HCT116 and HCT116 3-6 cells; however, p53 remained at high levels for a much longer time specifically in HCT116 3-6 cells. This response was similar to that observed in these MMR-proficient cells after exposure to ionizing radiation (5). Additionally, the levels of the p53-respon-
Three major sites of phosphorylation have been identified; phosphorylation of Thr14 or Tyr15 inhibits p34CDC2 activity, whereas phosphorylation of Thr161 is required for p34CDC2 activity (49–51). Thus, an antibody that specifically recognizes phosphorylated tyrosine was used to determine whether immunoprecipitated p34CDC2 was phosphorylated. Extensive tyrosine phosphorylation would indicate that cells had not yet entered mitosis, whereas the absence of tyrosine phosphorylation would indicate the cells were in mitosis. The tyrosine phosphorylation of immunoprecipitated p34CDC2 was prolonged in HCT116 3-6 cells compared with HCT116 cells (Fig. 6B). These data indicate that the majority of FdUrd-exposed HCT116 3-6 cells had not entered mitosis and thus were arrested in the G2 phase. In contrast, HCT116 cells showed the same initial G2 responses but progressed past this checkpoint more rapidly.

**MLH1-corrected HCT116 3–6 Cells Have a Greater Amount of FdUrd-induced DSBs.** The formation of DSBs has been thought to be responsible for FdUrd-mediated cytotoxicity (52, 53). Additionally, DSBs may be responsible for the induction of p53 and/or the G2 arrest seen with FdUrd treatment (54, 55). HCT116 and HCT116 3-6 cells were treated with various doses of FdUrd continuously for 72 h, then DSB formation was assayed using PFGE (Fig. 7). In both cell lines, treatment with as little as 2.5 μM FdUrd resulted in the appearance of DNA fragments. However, the degree of fragmentation was more pronounced in MMR-proficient HCT116 3-6 cells. In terms of DNA structure, the fragmentation pattern may be informative: mammalian replicons are ~0.1 Mb, which are clustered together to form minibands (1–2 Mb) and bands (as large as 6 Mb; Refs. 56 and 57). DNA fragmentation appeared within 48 h of treatment (not shown) and was completely blocked by the coadministration of dThd but not Urd (Fig. 7B). Similar results were observed after 5-FU treatment. DNA fragmentation was also greater in HCT116 3-6 cells treated with 6-TG (not shown). Additionally, the DNA fragmentation does not appear to simply reflect apoptosis; both HCT116 and HCT116 3-6 cells treated in this manner had identical, low levels of apoptosis (7.5 ± 0.5%) by a modified TUNEL assay. Thus, the enhanced formation of DSBs in HCT116 3-6 cells compared with HCT116 cells did not appear to be attributable to increased apoptosis in HCT116 3-6 cells.

**DISCUSSION**

Cells deficient in MMR are clearly more resistant to the cytotoxic effects of 5-FU and FdUrd (Figs. 1 and 2). Interestingly, it takes a long exposure time to FPs for this difference to become apparent in human cells, whereas only a short treatment time is necessary to see a similar effect in mouse cells. The reason for this is unclear; it may simply reflect differences in pyrimidine metabolism between human and mouse cells or between epithelial cells and fibroblasts. MMR-deficient cells also exhibit a less dramatic or less prolonged G2 arrest observed in these cells seems to reflect damage tolerance, which is a hallmark of MMR deficiency. The more prolonged G2 arrest observed in these cells may reflect the contribution of MMR in the processing of FP-induced DNA damage. Carethers et al. (34), did not report a difference in G2 response between HCT116 and HCT116 3-6 cells subsequent to 5-FU treatment; presumably, they examined cell cycle responses only after shorter exposures with higher doses of 5-FU. Although the enhanced G2 arrest observed in HCT116 3-6 cells at lower doses seems to have a negligible effect on survival, we believe it reflects the recognition of DNA damage by MMR, and that this arrest may be very important for mutation avoidance. At higher FP doses, we also observed equivalent G2 arrests between the cell lines; this may reflect the involvement of other types of DNA repair mechanisms.
repair, such as BER, in signaling the G₂ arrest. Additionally, whereas human HCT116 cells have wild-type p53, the apparent p53 mutation in the immortalized CT-5 and ME-10 mouse cells may contribute to the much greater degree of G₂ arrest seen in ME-10 cells by negating the G₁ arrest.

The FP-mediated G₂-M arrest, as determined by propidium iodide staining, was shown to be a true G₂ arrest (Fig. 5). This was expected, as asynchronous cells treated continuously with 0 to 10 μM FdUrd and 100 μM FdUrd; HCT116 3-6; HCT116 3-6 + 2.5 μM FdUrd). Data, mean ± SE.

Our data indicate that MMR may have a role in the recognition of maintaining the G₂-M checkpoint using HCT116 cells with targeted deletions of p53 and p21WAF1/CIP1 (59). We are currently investigating the role of p21WAF1/CIP1 in FdUrd-mediated MMR-dependent G₂ arrest responses. Other candidate effectors of a p53-mediated G₂-M arrest may also be involved, including the signaling adaptor protein 1-3-3p and GADD45 (47, 60); it is intriguing that p53 might mediate a G₂ arrest via GADD45 in this system. The levels of apoptosis induced by FdUrd exposure, as measured by a modified TUNEL assay, were relatively low (7.5%) in either cell line. Finally, HCT116 3-6 cells displayed a greater degree of DNA strand breakage subsequent to FdUrd treatment (Fig. 7). Although FPs have been reported to cause DNA breaks independently of MMR (52, 61), the increase in DSB formation may reflect the contribution (or interference) of MMR in processing DNA damage induced by FP incorporation or subsequent nucleotide pool imbalances.

We propose the model shown in Fig. 8 to represent how MMR mediates cellular responses to FP treatment. Briefly, the FP metabolite FdUTP can serve as a substrate for DNA polymerase. Thus, FP moieties can be incorporated into DNA and serve directly as substrates for MMR. The presence of FP moieties in DNA is not mandatory in this model, because the pleiotropic actions of 5-FU and FdUrd on pyrimidine metabolism can also result in dNTP pool imbalances as well as incorporation of uracil moieties into DNA. The misincorporation of naturally occurring bases by an error-prone DNA polymerase operating under suboptimal conditions would generate mispairs easily recognized by MMR. MMR, in conjunction with BER, would then process this damage. Because of the futile nature of this repair under continuous FP exposure, a prolonged G₂ arrest would occur, possibly in response to p53 and/or DNA strand breakage. Ultimately, the exacerbation of this situation by MMR may result in cell death.

In addition to the recognition and correction of simple mispairs of naturally occurring bases in DNA, MMR has been implicated in the detection and repair of other types of DNA damage. For example, MMR has been shown to bind DNA containing O⁶-methylguanine, the major lesion formed in DNA after treatment with monofunctional alkylating agents such as MNNG and temozolomide (62, 63). Upon replication, an O⁶-methylguanine:thymine mispair frequently occurs. Although MGMT is able to remove the methyl group from guanine, the contribution of MMR in coping with alkylation damage is clearly seen, especially when MGMT activity is inhibited by O⁶-benzylguanine treatment. Likewise, the purine antimetabolite 6-TG ultimately can be incorporated into DNA and then modified to form S⁶-methylthiothymine. MMR is able to recognize S⁶-methylthiothymine paired with either its normal partner cytosine or with thymine. MMR detection of this lesion may even be more pivotal here, because S⁶-methylthiothymine is not a good substrate for MGMT (64).

We propose the model shown in Fig. 8 to represent how MMR mediates cellular responses to FP treatment. Briefly, the FP metabolite FdUTP can serve as a substrate for DNA polymerase. Thus, FP moieties can be incorporated into DNA and serve directly as substrates for MMR. The presence of FP moieties in DNA is not mandatory in this model, because the pleiotropic actions of 5-FU and FdUrd on pyrimidine metabolism can also result in dNTP pool imbalances as well as incorporation of uracil moieties into DNA. The misincorporation of naturally occurring bases by an error-prone DNA polymerase operating under suboptimal conditions would generate mispairs easily recognized by MMR. MMR, in conjunction with BER, would then process this damage. Because of the futile nature of this repair under continuous FP exposure, a prolonged G₂ arrest would occur, possibly in response to p53 and/or DNA strand breakage. Ultimately, the exacerbation of this situation by MMR may result in cell death.

In addition to the recognition and correction of simple mispairs of naturally occurring bases in DNA, MMR has been implicated in the detection and repair of other types of DNA damage. For example, MMR has been shown to bind DNA containing O⁶-methylguanine, the major lesion formed in DNA after treatment with monofunctional alkylating agents such as MNNG and temozolomide (62, 63). Upon replication, an O⁶-methylguanine:thymine mispair frequently occurs. Although MGMT is able to remove the methyl group from guanine, the contribution of MMR in coping with alkylation damage is clearly seen, especially when MGMT activity is inhibited by O⁶-benzylguanine treatment. Likewise, the purine antimetabolite 6-TG ultimately can be incorporated into DNA and then modified to form S⁶-methylthiothymine. MMR is able to recognize S⁶-methylthiothymine paired with either its normal partner cytosine or with thymine. MMR detection of this lesion may even be more pivotal here, because S⁶-methylthiothymine is not a good substrate for MGMT (64).

Our data indicate that MMR may have a role in the recognition of
FP metabolites incorporated into DNA as well. The fact that FdUrd-induced cytotoxicity is completely reversed by dThd cotreatment argues that this cytotoxicity is DNA-directed, i.e., attributable either to FdUrd incorporation into DNA or to TS inhibition. Exogenous dThd was able to rescue this cytotoxicity because dThd can be converted sequentially by dThd kinase and nucleoside monophosphate and diphosphate kinases into dTTP, which then competes with dUTP for utilization by DNA polymerase α and also restores dTTP levels depleted by TS inhibition. FP metabolites can be incorporated into DNA, albeit at very low levels. For example, Kufe et al. (65) estimated that the ratio of [3H]FdUrd to [3H]dThd in the DNA of L1210 murine leukemia cells treated with equimolar (1 μM) concentrations of each nucleoside was 1.900. Parker et al. (66) found 8 nmol of [3H]FdUrd per mol of DNA nucleotide in the DNA of S-49 murine T lymphoma cells treated with 1.5 nmol of [3H]FdUrd for 12 h. Boothman et al. (67) found that treatment of HEP-2 cells with 0.1 μM FdUrd resulted in the incorporation of 1.83 × 10^-9 mol of FdUrd per mol of DNA phosphate. The actions of dUTP diphosphohydrolase, which guards against dUTP or FdUrd accumulation in the cell and the BER enzyme uracil-DNA-glycosylase, which efficiently removes uracil or 5-FU from DNA, are thought to be primarily responsible for this small degree of incorporation (68). However, actual demonstration of these pathways in vivo have been difficult. We propose that MMR is a major pathway that restricts the incorporation of 5-FU or uracil moieties into DNA.

Being a dThd analogue, FdUrd has been reported to pair with adenine as well as mispair with guanine moieties in DNA (69, 70). It is not known whether MMR can detect either of these pairings. Because the atomic radius of F is similar to H (1.35 Å compared with 1.2 Å), the DNA lesion caused by FP incorporation into DNA would be expected to be more subtle than the bulkier lesions created by MNNG or 6-TG treatments. However, studies with oligodeoxyribonucleotide duplexes containing FdUrd have revealed that this substitution does affect DNA structure. Duplexes containing dA:FdUrd bp were examined by NMR spectroscopy and found to have a greater rate of bp opening than dA:dT bp (71). dG:FdUrd bp engage in an equilibrium between wobble bp geometry and an ionized bp (72). FdUrd substitution affects the base roll angle at the site of FdUrd substitution, causing the helical axis of FdUrd-substituted DNA duplexes to be bent compared with nonsubstituted duplexes (73). Thus, MMR may directly recognize FdUrd mispairs in DNA, it may detect subtle distortions in the DNA helix caused by the presence of FdUrd, or it may even detect abasic sites in DNA created by the action of uracil-DNA-glycosylase.

The ability of dThd to rescue FdUrd-mediated cytotoxicity is also consistent with the notion that TS inhibition may be involved. TS has an essential, if not rate-limiting, role in DNA synthesis. It catalyzes the conversion of dUMP to dTMP using CH2=THF as the methyl donor. FdUMP forms a ternary, covalent complex with TS and CH2=THF, thereby blocking the pathway for de novo dTMP synthesis and thus causing dTTP levels to decrease (14). Importantly, alterations in the levels of any one of the dNTPs usually affect the levels of the other dNTPs by feedback regulation. For example, the inhibition of dTMP synthesis results in the lowering of dGTP as well as dTTP levels, whereas the dCTP, dATP, and dUTP levels increase. Conversely, the addition of excess dThd usually elevates dTTP, dATP, and dGTP levels but decreases the dCTP pool (74). Any perturbations in dNTP levels affect the fidelity of DNA replication.

It is well established that the probability that a correct or incorrect nucleotide is used by DNA polymerase α is related to the ratios of competing substrates (74). For example, when equal concentrations of the four dNTPs were provided in an in vitro DNA replication assay designed to detect base substitutions that revert an opal termination codon, the revertant frequency at this locus was found to be about 1 × 10^-5. However, if one of the dNTPs was present at a 20-fold excess, the rate of stable misincorporation increased to 1.4 × 10^-4 (75). Furthermore, variation in the absolute concentration of dNTPs strongly affects replication fidelity by altering the balance between incorporation of the next correct nucleotide versus exonucleolytic removal of an improper nucleotide just incorporated (76). Fluctuations in dNTP concentrations can also increase frameshift error rates at homopolymeric runs, perhaps by leading to polymerase dissociation with subsequent misaligned reassocation (77, 78). In addition, inhibition of TS by FdUMP leads to increases in dUTP levels and hence an increase in dUTP incorporation into DNA (79, 80). Thus, dNTP pool perturbations caused by FdUrd treatment would lead to the generation of DNA mismatches because of an increase in the DNA polymerase error rate. The MMR machinery would then become involved as it detects these mismatches and perhaps detects dUrd in DNA as well. In fact, MLH1 has been reported to interact with methyl-CpG-binding endonuclease 1/5-methylcytosine binding domain 4, which acts as a mismatch-specific glycosylase active on thymine, uracil, and 5-FU paired with guanine moieties in DNA (81). HCT116 cells expressed this enzyme, but were found to have a methyl-CpG-binding endonuclease 1/5-methylcytosine binding domain 4 mutation in one of their alleles (82).

DNA single- and double-strand breaks have been observed in various cell lines after FP treatment and are considered important determinants of FdUrd cytotoxicity (52, 53, 61, 83, 84). DSBs elicit G2 arrest responses in eukaryotic cells (85, 86), and DNA repair during this checkpoint is thought to be needed before chromosome segregation (41). The creation of DSBs or G2 arrest responses does not appear to be the result of apoptotic response differences between MMR-proficient and MMR-deficient cells. Instead, our data suggest that MMR may directly signal a G2 arrest to allow repair to occur. Alternatively, MMR may indirectly signal an arrest through the generation of DSBs after FdUrd exposures. DSBs may be the result of futile cycles of repair of FdUrd or dUrd moieties in DNA, performed by MMR in concert with BER. According to this theory, the MMR machinery (upon recognition of a mismatch) would excise a long patch of nucleotides (on the order of 1 kb in length) from the strand containing the inappropriate nucleotide(s) (87, 88). Upon resynthesis of this strand, even more FdUrd and dUrd nucleotides would be incorporated into the new strand of DNA. Furthermore, concurrent dNTP pool imbalances would lead to misincorporations of the naturally occurring nucleotides. This would prompt an even more extensive cycle of repair. Perhaps because of fragility created simply by the magnitude of this repair, the interference from other DNA repair enzymes (e.g., glycosylases), the stalling of the DNA polymerase, or the simple overlap of repair on opposing strands, DSBs are created. Whereas this futile repair would occur to some extent (because of BER) in MMR-deficient cells, the contribution of an intact MMR system would greatly worsen this situation. This DNA damage could then signal (perhaps involving p53 or p73) a cell cycle arrest and/or cell death via apoptosis (89, 90). However, the exact mechanism by which MMR-mediated DSBs may occur after only one round of DNA replication, especially apparent in the G2 arrest responses observed in synchronized HCT116 3-6 compared with HCT116 parental cells, remains to be determined.

This study suggests that FdUrd-mediated DNA lesion detection by MMR is part of a major DNA repair mechanism within the cell for maintaining genomic stability after pool imbalances caused by FdUrd exposure, or by incorporation of FdUrd or dUrd moieties into DNA. These observations have direct clinical relevance because nearly 15% of all colon cancer may be attributed to the loss of MMR. FP antimetabolites remain standard drugs used for the treatment of colon
cancer. Many combined treatment modalities have been designed to increase DNA-level cytotoxicity of 5-FU (e.g., by increasing the production of FdUMP and/or the stability of FdUMP-TS) with the anticipation and, to some extent, the realization of increased tumor-selectivity. The data presented in this paper suggest that such DNA-targeted cytotoxicity may be contraindicated when treating the tumors of hereditary nonpolyposis colorectal cancer patients. In these circumstances, increased DNA-level cytotoxicity may result in increased resistance to drug treatment attributable to damage tolerance in tumors that lack MMR activity. Furthermore, MMR-deficient cells could, in fact, be selected for by such DNA-directed FP treatment. In addition, such resistant tumors may have greatly increased rates of genetic instability because of FP-induced DNA lesions, which are MMR-resistant. This condition could increase genetic instability, resulting in increased tumor heterogeneity and selection for more malignant and invasive tumor cells.

ACKNOWLEDGMENTS

We thank Drs. C. Richard Boland, Andrew Buermeyer, and R. Michael Liskay for providing cells, and Dr. Tom Davis, Mike Sramkoski, Dr. James Jacobberger, and Dr. Antonio Gualberto for their help during the course of these investigations.

REFERENCES

Role of the hMLH1 DNA Mismatch Repair Protein in Fluoropyrimidine-mediated Cell Death and Cell Cycle Responses

Mark Meyers, Mark W. Wagner, Hwa-Shin Hwang, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/13/5193

Cited articles
This article cites 86 articles, 51 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/13/5193.full.html#ref-list-1

Citing articles
This article has been cited by 37 HighWire-hosted articles. Access the articles at:
/content/61/13/5193.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.