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Title: **ROLE OF CELL CYCLE REGULATION AND MLH1, A KEY DNA MISMATCH REPAIR PROTEIN, IN ADAPTIVE SURVIVAL RESPONSES**

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Principal Investigator **David A. Boothman, Ph.D.**

Contractor's Name and Address **University of Wisconsin-Madison**
Madison, Wisconsin 53792

Present Address: **Department of Radiation Oncology**
10900 Euclid Avenue (BRB-326 East)
Case Western Reserve University
Cleveland, Ohio 44106-4942
Tel.: (216) 368-0840
Fax: (216) 368-1142
e-mail: ***dab30@po.cwru.edu***

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Role Of Cell Cycle Regulation and MLH1, A Key DNA Mismatch Repair Protein, In Adaptive Survival Responses

I. INTRODUCTION: Due to several interesting findings in our laboratory on both adaptive survival response (ASRs) and DNA mismatch repair (MMR), we separated this grant into two discrete Specific Aim sets (each wi their own discrete hypotheses). The described experiments were simultaneously performed. In the first set Specific Aims, we examined the role of PCNA, cyclins A and D1, cdk2, and xips5 & 13 (once cloned) in the G_0/C cell cycle regulation checkpoint, which occurs in certain human cancer cells during ASRs caused by low do ionizing radiation (IR) exposures. These experiments entailed cloning xips5 & 13, and then examining the role these x-ray-inducible proteins (xips), along with specific cell cycle regulatory proteins (i.e., PCNA, cyclins A ar D1, and cdk2), in ASRs. These experiments were performed as a direct continuation of our recent past work this DOE grant.

In the second set of Specific Aims, we examined the role of the human DNA mismatch repair protei hMLH1, in G_2/M cell cycle checkpoint arrest, which occurs following IR, 5-fluoro-2'-deoxyuridine (FdUrd), or thioguanine (6-TG) exposures; FdUrd and 6-TG were used as positive controls for MMR detection ar subsequent responses. Our goals in these experiments were to further explore the apparent dual role of th hMLH1 protein in both MMR and G_2/M cell cycle checkpoint regulation. We also examined the potenti interactions of hMLH1 in ASRs, thus unifying the two sets of Specific Aims. Finally, we have completed ou studies on the role of DNA-PK in ASRs, and found that this DSB repair complex does not play a role in ASRs.

The following Speciufic Aims were explored:

Specific Aim #1 (A1): To determine the effects of altered regulation of cyclins A & D1, PCNA, an cdk2 on ASRs in human normal compared to cancer cells (Years 0-3).

Specific Aim #2 (A2): To clone xips 5 & 13 using unique DNA sequences previously determined (1 produce antibodies to each of the proteins, and then determine the effects of altered regulation of xips & 13 proteins on ASRs in human cancer compared to normal cells (as described in Specific Aim #1 (Years 0-3).

Specific Aim #3 (A3): To determine the effects of altered hMLH1 expression on ASRs occurring i human and rodent cells (Years 0-1).

Specific Aim #4 (A4): To determine whether DNA mismatch repair (MMR) in general, or the hMLH protein specifically regulates G_2/M cell cycle checkpoint arrest (Years 0-3).

Specific Aim #5 (A5): To determine if the loss of the hMLH1 protein (or hMSH2 protein) alters G_2/M G_0/G_1, and pinpoint the exact G_2/M cell cycle checkpoint arrest which occurs differentially in parent (wild-type) or hMLH1-proficient compared to hMLH1-deficient cells (Years 0-3).

Specific Aim #6 (A6). To further delineate the cell cycle position of hMLH1-proficient compared hMLH1-deficient cells after IR, 6-TG or FdUrd treatment (Years 0-1).

Specific Aim #7 (A7): To determine what proteins interact with the hMLH1 protein, and to determir if these hMLH1-protein complexes control G_2/M or G_0/G_1 cell cycle checkpoints (Years 0-3).

III. PROGRESS REPORT-SUMMARY OF PUBLISHED DATA.

Progress on Specific Aims #1-3.

Characterization of ASRs In Human Normal and Neoplastic Cells. Human normal and neoplastic cel were exposed to low "priming" doses of IR (5 cGy/day) over a 4 day period. Primed and unprimed (untreate mock-irradiated) cells were then exposed to a high "challenging" dose of IR on day 5. Challenging doses we selected to give equitoxic survival levels (20% survival with no time for recovery) for each cell type (Table 1 Boothman et. al., (1)). Low dose primed U1-Mel and HEp-2 cells demonstrated 2.3- to 2.4-fold surviv enhancement over their unprimed counterparts following a high dose challenge as measured by colony-formir ability (U1-Mel survival increased from 24% to 56% and HEp-2 survival increased from 17% to 41%). Enhance survival due to low dose IR was prevented by 4 hr transient post-irradiation, 5 μg/ml actinomycin D (data n shown) or 10 μg/ml cycloheximide treatments, indicating that both new transcription and protein synthesis we

required for the emergence of the more radioresistant phenotype. In contrast, survival enhancement responses we not observed in normal human fibroblasts or in HTB-152 neoplastic cells at any priming dose tested (1, 18).

Influence of Priming Doses on ASRs. We previously noted that differences in transcriptional responses we dose-dependent (2, 14, 114), so we investigated the possibility that different cells required different priming dos for ASRs. Confluence-arrested human U1-Mel, HEp-2, and GM2936B cells were treated with various primir doses of IR, challenged or not with a high dose of radiation, and then treated with or without CHM (10 μg/ml) f 4 hr post-irradiation. Significant ASRs were seen in U1-Mel and HEp-2 cells using a range of priming dos between 1-10 cGy/day X 4 days. In contrast, we failed to note significant ASRs in GM2936B cells using primir doses of 0.1-0.5 cGy/day X 4 days.

We then investigated the longevity of ASRs. Cells were primed as before using either 5 cGy/day x 4 da for U1-Mel and HEp-2 cells or 0.5 cGy/day x 4 days for GM2936B cells. Primed cells were then exposed to a equitoxic high dose challenge at various times (1-5 days) thereafter to determine how long the ASR could b maintained. No ASRs were noted in normal human fibroblasts. In U1-Mel and HEp-2 cells the observed ASF steadily decreased with time (in days) and were prevented by 4 hr transient post-irradiation treatment with 10 μg/n CHM. By 5 days after the last priming dose, no ASRs were observed.

Dose-Response Effects On ASRs. Confluence-arrested U1-Mel, HEp-2, and GM2936B cells were treated wi or without priming doses of 5 cGy/day X 4 days; GM2936B cells were also treated with 0.5 cGy/day X 4 day Cells were then challenged with various high doses of IR to generate dose-response curves. Survival Enhancemer Ratios (SERs), analogous to Dose Enhancement Ratios (115-117), were then calculated. U1-Mel and HEp-2 cel exhibited SERs of 2.0 and 2.2, respectively, after cells were primed with 5 cGy/day X 4 days. As expecte GM2936B cells demonstrated no ASR at 0.1-5.0 cGy/day X 4 days. Post-IR exposure to 10 μg/ml CHM blocke ASRs in U1-Mel or HEp-2 cells. In our recent paper (1) we summarized a series of experiments in whic various confluence- and low serum-arrested human cells were examined for ASRs at various priming doses. Onl U1-Mel and HEp-2 human cancer cells demonstrated ASRs at specific optimal priming doses. Human norm fibroblasts did not show ASRs. These descriptive data developed over the course of the first six years of this gra have now allowed us to examine primed and challenged cells for molecular markers of ASRs, which act in DN repair and/or the establishment of ASRs by regulation of the cell cycle at the G_0/G_1 checkpoint.

Flow Cytometric and Labeled Nuclei Assessments. We determined the cell cycle distribution of confluenc and low serum-arrested human cells 4 hr after IR using flow cytometry and measured of [^{3}H]thymidine-labele nuclei (1). Throughout the ASR treatments, human normal and neoplastic cells remained in G_0/G_1 (>95% of t cells were in G_0/G_1 by flow cytometry); their population distributions were not affected by low or high dos challenges. We also replated primed and unprimed cells to determine their plating efficiencies and to monitor the re-entry into the cell cycle (1). Both primed and unprimed cells of all lines were >93% viable. From the experiments, we noted that primed U1-Mel or HEp-2 cells entered S phase (as measured by increases [^{3}H]thymidine incorporation and cell number) within 6-8 h, whereas unprimed cells entered S phase in 12-14 depending upon cell type, after low density replating (1).

Identification of Transcripts Whose Levels Were Altered During ASRs In U1-Mel Cells. To identi molecular markers of ASRs in human cells, we examined previously identified and isolated xips1-12 (4) transcri levels for alterations during low priming and high dose challenging exposures of IR (i.e., 450 cGy) in human U Mel cells (1). We also examined alterations in other transcripts, including cyclins A,B,D1 (Prad 1), cdc glutathione-S-transferase-π, thymidine kinase, small proline rich protein, DT Diaphorase, tissue-type plasminoge activator, c-fos, c-jun, transforming growth factor-ß (TGF-ß), p53, and several loading standards, including 36B ß-2-microglobulin, and actin. Primed and unprimed U1-Mel cells were also treated with or without 10 μg/n CHM or 5 μg/ml actinomycin D for 4 hr immediately after each priming or high dose challenge and total RN was subsequently extracted. Changes in transcript levels were measured via scanning densitometry (1, 4, 18) ar normalized for loading variations using 18S and 28S rRNA levels from the ethidium bromide stained gels, 36B levels, or actin or ß-2-microglobulin levels as loading standards to calculate relative mRNA abundance levels described (1, 2, 4).

Transcripts hybridizing to xip5 (~7 kb) gradually accumulated after each 5 cGy priming dose of radiatic in growth-arrested human U1-Mel or HEp-2 cells. Likewise, a 0.5 kb transcript related to xip12 (hereafter referre to as xip13) gradually increased in response to low priming doses of radiation (1). The transcript corresponding the original xip12 cDNA clone (~10 kb) did not change, except in response to a high dose of radiation, and th

levels of expression of this gene were not different between primed and unprimed cells receiving 450 cGy (data n shown). Transcript levels of both xips5 & 13 in primed cells following a high dose challenge were not as high those observed in unprimed U1-Mel cells which had been challenged with a single high dose (450 cGy) of I This may signify negative feedback control of gene expression due to low dose exposures. Treatment of U1-M or HEp-2 cells with actinomycin D for 4-5 hr after each exposure to radiation prevented expression of xips5 ar 13 during priming and high doses of radiation (1, 4). These data indicate that new gene transcription is responsib for dramatic increases in xips5 and 13 during ASRs and suggest that new transcription and translation of protein required for ASRs. Interestingly, we also demonstrated that new changes in any of the more common transcriptic factors did not occur during ASRs, suggesting alterations in more novel transcription factors are likely to take pla which regulate ASRs (3).

We also examined the expression of other transcripts which were known to be cell cycle-regulate implicated in DNA repair, or induced by IR. Cyclin transcripts were examined since many X-ray-inducib proteins (12) and transcripts (4) from U1-Mel cells were also cell cycle-regulated (Boothman et. al., unpublishe data), and changes in cyclin B transcript and protein levels were reported following acute doses of radiation HeLa cells (19). We found that cyclin A transcripts accumulated during low priming doses (1). It is interestir that changes in the levels of cyclin A were not observed in unprimed U1-Mel cells exposed only to a hig challenging dose of radiation. Cyclin D1 (Prad 1) transcript levels also increased after IR; however, its levels di not continually increase with each priming dose, and similar levels were observed after 5 or 450 cGy. Like xips & 13, levels of glutathione-S-transferase-π gradually increased following low doses of IR. After correcting f loading variations, cyclin A, cyclin D1, and GST-π increased 300%, 200%, and 50%, respectively, in primed cel exposed to 450 cGy compared to unprimed cells treated with 450 cGy. Similar transcript responses describe above were found in HEp-2 cells during ASRs (1).

Several other transcript levels were either not affected by low priming doses of radiation or were nev expressed under any of the conditions used, except in log-phase control cells. Differences in xips1,2,3,4,7,8,9,1 & 12, cyclin B1, DT diaphorase, cyclophilin, gelatinase, stromelysin, 36B4, actin, ß-2-microglobulin, tissue-ty plasminogen activator, p53, c-fos, c-myc, and egr-1 were **not** noted between primed and unprimed cells, or aft challenge with a high dose of IR (1). Transcripts which were not expressed in the ASR experiments describe above, but were expressed in log-phase U1-Mel cells, included: thymidine kinase, cdc2, cyclin B1, TIMP-1, ar collagenase-1. Similar transcript responses described above were also found using HEp-2 cells. xip8 prote levels, which bind to Ku70 and may be a crucial factor for cell death after IR treatment (Yang et. al., Natu Medicine), was expressed in confluence-arrested cells and turned off on log-phase cells.

Protein Expression Analyses Before and After ASRs in HEp-2, U1-Mel, Normal Human Fibroblast and Other Human Cell Lines. We then characterized molecular changes in proteins during ASRs in hum cells. More specifically, we concentrated on the human neoplastic HEp-2 and U1-Mel cells compared to norm human fibroblasts, GM2936B, GM2907A, and GM2937A cells. We repeated and confirmed the transcription up-regulation of cyclin D1, cyclin A, xips5, & 13 in HEp-2 and U1-Mel cells (above). However, the transcriptional increases were not observed in any of the normal human fibroblasts examined. Furthermore, we di not observe increases in radiation resistance to a high dose challenge after low dose IR adaptation in normal hum fibroblasts. *Therefore, to date we have not observed ASRs in normal human cells. The only ASRs we ha observed were in human U1-Mel and HEp-2 cells* (1). Why were U1-Mel and HEp-2 cells able to produce ASR while other normal human fibroblasts were not? In the next three years, we will examine specific gene produc which we believe may regulate ASRs in HEp-2 and U1-Mel cells, and investigate their role(s) in ASRs in U1-M and HEp-2 cells compared to normal cells. We have completed experiments with other cell models, such various scid mouse cells +/-DNA-PK, and human HCT-116 cells +/-hMLH-1 gene product, in order to investiga the role of specific gene products in ASRs in human cells. We have eliminated DNA-PKcs as playing a role ASRs in rodent cells using a comparative analysis of scid and CB17 cells (118).

To investigate the potential role of cyclin A, cyclin D1, PCNA, pRb (retinoblastoma protein), WAI 1/CIP1/p21, mdm-2, and p53 proteins in ASRs in human HEp-2 and U1-Mel cells compared to various norm human fibroblasts (GM2937A, GM2907A, and GM2936B), we examined Western immunoblot ar Immunoprecipitation/Western immunoblot analyses on unirradiated, low dose primed (1-5 cGy X 4 days), lo dose primed and high dose challenged (1-5 cGy X 4 days + 300-450 cGy) compared to high dose challenge alone (300-450 cGy) U1-Mel cells. Analyses of our data have demonstrated some surprising conclusions.

First, the increases in cyclin D1 and PCNA transcripts previously observed (above) did **not** result apparent increases in protein levels (Boothman et. al., Mutat. Res., 1996). On the other hand, we were surprised find extremely high, constitutive levels of cyclin D1 and PCNA levels (20- to 50-fold) in U1-Mel and HEp-2 cel

compared to normal human fibroblasts. Since U1-Mel and HEp-2 were the only cell lines to demonstrate ASR we concluded from these data that increases in cyclin D1 and PCNA transcripts after low doses of radiation whic confer ASRs were insignificant compared to the elevated endogenous protein levels of PCNA and cyclin A with U1-Mel and HEp-2 cells prior to low level radiation. *These data indicate that PCNA and cyclin D1 protein leve were not altered after low level IR, and that other proteins, which make up this DNA repair complex, mu increase after low level radiation in order for this DNA repair complex to confer ASRs in certain human cell* Western blot analyses of HEp-2 and U1-Mel cells during ASRs and after high dose challenges demonstrated r alterations in PCNA levels after low or high dose challenging events, and there were no increases in protein leve during ASRs. PCNA levels were, however, greatly elevated in U1-Mel and HEp-2 cells compared to norm human fibroblasts and the human colon cancer cell line HCT-116. PCNA levels were not altered by low or hig dose IR and we, therefore, concluded that elevated levels of PCNA may be important for ASRs, but are **not** a maj controlling factor.

Similar results, surprisingly, were discovered for cyclin D1. Cyclin D1 levels in arrested cells have bee reported to be extremely low (119). In HEp-2 and U1-Mel cells, however, their levels were remarkably elevate compared to normal human fibroblasts and HCT-116 cells, both of which do not demonstrate ASRs. We four that cyclin D1 levels, as for PCNA (above), were constitutively present at very high levels in U1-Mel and HEp- cells. Retinoblastoma (pRb) and Topo I protein levels were also not affected by low or high dose IR, as previous described (120). We are currently examining changes in the phosphorylation level of the pRb protein before ar after IR to further pinpoint the position of cell cycle regulation during ASRs. Similar results were found in HEp- cells using either Western blot or immunoprecipitation analyses, in which Western immunoblot analyses are the used to demonstrate specific protein levels. We also examined p53 and cdk2 levels during ASRs and after hig dose challenges. No alterations in p53 or cdk2 levels were noted during low doses (5 cGy) of IR. As recent described (121), p53 levels in human U1-Mel cells were induced by high doses of radiation. A similar lack cdk2 protein responses with HEp-2 cells were also observed. These data suggest that cdk2 and cyclin D1 protei levels do not change, but do not necessarily mean that cyclin-dependent cdk2 enzyme levels are not altered aft low dose IR. This is currently being examined in our laboratory.

As indicated above, we also examined changes in cyclin A transcript and steady state protein levels usir Western and immunoprecipitation blotting analyses before and after low ASR radiation doses compared to hig doses of IR. Unlike the responses found above for cyclin D1 and PCNA, cyclin A levels were not present befo low dose priming. During low dose priming, cyclin A levels dramatically increased as observed by Western bl and immunoprecipitation analyses (Boothman et. al., in preparation). Since this protein increased at both t transcript and translational levels following low doses of IR, it is a likely candidate for controlling ASRs in certa human cells. These data, in combination with our data demonstrating elevations in PCNA and cyclin D1 protei levels described above using U1-Mel and HEp-2 cells, suggest that all three protein levels (at least) must be prese at one time to comprise a DNA repair complex. This complex may be composed of cyclin D1 and PCNA (whic are **constitutively** elevated in HEp-2 and U1-Mel cells), and cyclin A. Cyclin A may be the controlling protei which is inducible or whose expression increases due to altered cell cycle regulation following repeated low dos of IR. Cyclin A may control ASRs through apoptotic inhibition mechanisms, or it may in turn control (activat other proteins which participate in ASRs in certain human cells. We will explore the role of cyclin A in ASF using doxycycline-inducible, cyclin A expression plasmids as described in the first set of Specific Aims of th current DOE grant renewal. ASRs will be analyzed in U1-Mel and HEp-2 cells (which have elevated PCNA ar cyclin D1 levels) with or without sense/antisense cyclin A expression. These experiments will test the hypothes that ASRs are regulated by cyclin A levels. Endpoints measured will included colony forming abilities (for ASRs sister chromatid exchanges, apoptosis [by morphology, pol(ADP-ribose) polymerase cleavage, and flo cytometry], and cell cycle population analyses via flow cytometry. We will also analyze cyclin A, cyclin D1, ar PCNA transcript and protein levels via Northern blot analyses, Western blot analyses, and immunoprecipitatio respectively.

Progress on Specific Aims #4-7.:

Summary of Cell Cycle Regulation of DNA Mismatch Repair Proteins. HNPCC is a cancer susceptibili syndrome caused by mutations in several genes involved in MMR, including *hMSH2*, *hMLH1*, or *hPMS2*. Rece reports suggested that hMSH2 and hMLH1 have a role in the regulation of the cell cycle (7, 40, 122). T determine if these genes are cell-cycle regulated, we examined their mRNA and protein levels throughout the ce cycle in synchronized IMR-90 normal human lung fibroblasts (5). We recently demonstrated that the levels hMSH2 mRNA and protein do not change appreciably throughout the cell cycle. In contrast, while hMLH mRNA levels remain constant, a modest (~50%) increase in its protein levels during late G_1- and S-phases of t cell cycle occurred. The levels of hPMS2 mRNA fluctuated mildly (decreasing 50% in G_1 and increasing 50% :

S phase), while hPMS2 protein levels increased 50% in late G_1- and S-phases of the cell cycle. Our data indica that, at least in synchronized normal human primary fibroblast cells, the machinery responsible for detection ar repair of mismatched DNA bases is present throughout the cell cycle, with little modulation of hMLH1, hMSH or hPMS2 protein or mRNA levels (5).

Role of hMLH1 In G_2/M Cell Cycle Checkpoint Arrest.

MLH1 Expression Before and After IR. Two genetically matched human HCT116 colon carcinoma cell lin were used to determine whether MMR mechanisms played a role in survival after exposure to IR compared to TG. We also investigated whether specific proteins involved in MMR (i.e., hMSH2 or hMLH1) were damag inducible. The hMLH-1 protein was not expressed in parental HCT116 ($hMLH1^-$) cells, but was expressed HCT116 3-6 ($hMLH1^+$) cells. This represents the first demonstration that HCT116 cells do not express t hMLH1 protein. Further comparisons suggested that the overall expression level of hMLH1 in HCT116 3-6 cel was qualitatively similar to those found in normal IMR-90 human fibroblast cells (123). hMSH2 protein leve were not altered by the chromosome 3 microcell transfer, since similar levels were expressed in both HCT11 ($hMLH1^-$) and HCT116 3-6 ($hMLH1^+$) cells and levels of hMSH2 were comparable to those levels present IMR-90 normal human fibroblasts, as previously demonstrated (5).

We then examined the regulation of hMLH1 and hMSH2 proteins after IR. No changes in hMLH1 (8 kDa protein) or hMSH2 (100 kDa protein) levels were observed at 4-48 h after 5 Gy in HCT116 ($hMLH1^-$) HCT116 3-6 ($hMLH1^+$) cells. Levels of alpha-tubulin were used as a loading control, since they were not alter after IR (4, 12). These data suggest that hMLH1 and hMSH2 proteins were not altered by IR in either cell line. addition, the proteins do not appear to be cell cycle regulated, since significant changes in cell cycle distributic occurred over the course of the experiment following IR. These data are consistent with our recent cell cyc analyses of synchronized normal human IMR-90 fibroblast cells, in which hMLH1 and hMSH2 protein ar transcript levels remained constitutively expressed during cell cycle progression (5).

Effect of hMLH1 Expression Deficiency on Survival After IR or 6-TG. We examined differences sensitivities between HCT116 and HCT116 3-6 cells after IR (0-8 Gy) by limited dilution colony forming surviv assays. A statistically significant difference in survival [$p<0.049$, (40)] between HCT116 ($hMLH1^-$) and HCT11 3-6 ($hMLH1^+$) cells was found, with the former being modestly more sensitive to IR damage. We also observe the previously reported (113, 124) phenomenon of "damage tolerance" of mis-incorporated bases in HCT116 cel following growth in the presence of 6-TG. HCT116 3-6 cells demonstrated a dramatic sensitivity to this age (~50-fold), following 12 h pulses of 6-TG, in contrast to the reversed situation above with IR.

Effect of hMLH1 Deficiency on Cell Cycle Checkpoint Delays. We then examined differences in cell cyc checkpoint arrests between parental HCT116 and hMLH1-corrected HCT116 3-6 cells. HCT116 3-6 ($hMLH1^+$ cells demonstrated extensive G_2/M cell cycle checkpoint arrest responses following a 5 Gy dose of IR, with leve nearly twice that of parental HCT116 ($hMLH1^-$) cells given approximately equitoxic doses. The G_0/G_1 arrest w rather small in either cell line in the first 12 h. In addition, HCT116 3-6 cells arrested 12 to 24 h longer in t G_2/M phase of the cell cycle than HCT116 cells exposed to approximately equitoxic doses of IR. For example, 24 h following 5 Gy, 67% of irradiated HCT116 3-6 cells were in the G_2/M cell cycle phase, compared to 21 G_2/M cells in identically irradiated, parental HCT-116 cell population. The enhanced G_2/M arrest observed HCT116 3-6 cells was dose-dependent up to 8 Gy, when nearly all cells appeared to be arrested in this phase of t cell cycle. Interestingly, no differences in the overall S-phase cell population for up to 24 h were noted usir propidium dye staining and flow cytometric analyses before or after IR (or 6-TG, below) in HCT116 or HCT 11 3-6 cells. Thus, the dramatic G_2/M cell cycle arrest in HCT116 3-6 cells appeared to precede and preclude ar observable arrest at G_0/G_1. These data strongly suggest that loss of hMLH1 protein corresponded with a loss of competent, albeit not complete, G_2/M cell cycle checkpoint. The low degree of G_2/M cell cycle arrest observed HCT 116 cells may be caused by the generation of frank double strand breaks created by IR, at a poi independent of hMLH1-dependent G_2/M cell cycle arrest.

Previously, a 50-fold enhanced survival of HCT116 ($hMLH1^-$) over HCT116 3-6 ($hMLH1^+$) was reporte by Boland *et al.* (113, 122, 124, 125) following treatment of cells with *N*-methyl-*N'*-nitrosoguanidine (MNNG) 6-TG. Furthermore, it was shown that HCT116 3-6 ($hMLH1^+$) cells responded to these agents by arresting G_2/M, while HCT116 ($hMLH1^-$) cells continued to cycle normally. Using analogous conditions as tho

previously described (113, 122, 124) a similar difference in cell cycle distribution following 6-TG treatment wa observed. As previously noted (113, 122, 124), parental HCT116 cells demonstrated no change in the percentag of cells in G_2/M following 6-TG exposures. The G_2/M arrest observed in HCT116 3-6 cells following 6-T treatment was dose-dependent, but not for HCT116 cells. While both cell types demonstrated sub-G_0/C (apoptotic) peaks, a relative increase was consistently noted in the MMR-proficient HCT116 3-6 cells compared t the parental MMR-deficient HCT116 cells on day 6 (data not shown). The apoptotic responses observed were nc however, sufficient to explain the nearly 50-fold increase in lethality in the corrected HCT116 3-6 cells compare to HCT116 cells.

Effect of hMLH1 Deficiency on Damage-inducible Nuclear p53 Responses. Although parental and correcte HCT116 cells exhibited differences in radiation sensitivity and cell cycle alterations, both cell lines demonstrate similar IR-induced nuclear p53 levels following approximately equitoxic doses of IR. Interestingly, however, th kinetics of p53 induction, and specifically its loss over time following IR, differed significantly between the tw genetically match cell types. Evaluation of nuclear protein levels indicated that HCT116 3-6 cells maintaine elevated levels of p53 protein beyond 24 h following doses as low as 2 Gy. HCT116 cells, however, demonstrate an equivalently high initial level of p53 induction at 2-4 h at all doses examined, but p53 levels were more transie and waned by 12 to 24 h, in a dose-dependent manner. The elevation of p53 protein levels (~50-fold higher than non-irradiated cells) was more long-lived in HCT116 3-6 cells, even though the cell cycle distribution varied widel over the course of the experiment. Interestingly, similar increases in nuclear p53 levels were observed in log-pha and confluence-arrested HCT116 (hMLH1$^-$) or HCT116 3-6 (hMLH1$^+$) cells (data not shown). These da strongly suggest that the induction of p53 was in direct response to DNA damage rather than to secondary cel cycle changes. No correlation between p53 induction and the timing or overall extent of cells at the G_2/M cell cyc checkpoint arrest were noted.

We noted dramatic induction of both nuclear and whole cell p53 levels in the HCT116 3-6 (hMLH1$^+$ cells after 6-TG exposure, while no such increase was noted for MMR-deficient HCT116 (hMLH1$^-$) cells until dose of 6 μM was used, even after 6 days of continuous drug exposure. These results are consistent with a ro for p53 in response to DNA damage (26, 39, 126, 127) and strongly suggest that cells deficient in hMLH expression fail to recognize or process damage caused by 6-TG incorporation. The absence of p53 inductic following 6-TG exposure in HCT-116 (hMLH1$^-$) suggests a role of the hMLH1 protein in both the detection mismatch DNA base damage created by 6-TG, and in the intracellular signaling events which elicit the damag inducible p53 post-transcriptional process (36, 126, 128).

E6 Expressing Cells Demonstrate That Neither G_1 Arrest, Nor A Functional p53 Affect the hMLH1-Mediate G_2/M Cell Cycle Checkpoint Arrest. To directly determine the role of p53 in the G_2/M cell cycle checkpoint arre described above, we infected cells (HCT116 and HCT116 3-6) with retroviral expression vectors expressing th papillomavirus E6 protein. In addition, since it is possible from the data above that hMLH1 may play a role in C cell cycle arrest or transition into S-phase, in conjunction with p53, we wished to explore the effects of loss of C arrest on the cell cycle distribution effects following IR or 6-TG. The E6 protein is known to bind to, and targe p53 for ubiquination and proteolysis and results in a loss of G_1 cell cycle arrest induced by DNA damaging agen (129). The efficiency of E6 in removing p53 protein (even from IR-induced cells) was demonstrated. Loss of p5 did not affect the overall levels of hMLH1 protein expressed, nor alter the levels of this protein following II Following 5 Gy, E6 transfectants of both cell types demonstrated similar cell cycle kinetics compared nontransfected cells that retained intact p53 function. An exception was HCT116 E6 clones, which had a reduce G_0/G_1 arrest early after exposure and showed slightly greater G_2/M arrest than normal nontransfected ar irradiated HCT116 cells. The loss of p53 function also did not affect survival following IR exposure. These da strongly suggest that p53 does not play a role in G_2/M cell cycle checkpoint arrest, nor in the survival differenc observed between cells that express MLH1 (HCT116 3-6) or cells which have lost expression of this MM protein (HCT116 cells). These data, along with the lack of a difference in S-phase fractions between irradiated ar nonirradiated HCT116 and HCT116 3-6 cells, also strongly suggest that hMLH1 does not play a role in G_1 ce cycle checkpoint arrest or G_1-S transition.

Primary Embryonic Fibroblasts From MLH1 Knockout Mice Also Demonstrated Deficient G_2/M Cell Cyc Checkpoint Responses. Next, we wanted to demonstrate that the apparent hMLH1-dependent G_2/M cell cyc checkpoint arrest phenomena were not unique to HCT116 3-6 human colon carcinoma cells. Therefore, v performed identical cell cycle regulatory studies using fibroblasts from MLH1 knockout "C57BL/6J" (B6) blac

mice and compared their responses to genetically matched, primary embryonic murine fibroblasts derived fro parental wild-type mice before and after IR or 6-TG. In control experiments, we examined MLH1 and MSH protein expression in three representative clones: one homozygous wild type at the ($MLH1^{+/+}$) locus, or heterozygous ($MLH1^{+/-}$), and one homozygous mutant at this locus ($MLH1^{-/-}$). As expected, heterozygous ar homozygous wild-type cells expressed normal basal levels of both MLH1 and MSH2, at levels similar to tho previously found in IMR-90 cells (5). In contrast, homozygous mutant cells ($MLH1^{-/-}$) did not express the murir MLH1 protein, but did express normal levels of murine MSH2. Neither MMR protein levels appeared to va following 6-TG or IR treatments. Interestingly, p53 levels within all of these clones did not vary following IR or TG treatments, and remained constitutively high (data not shown). As in HCT116 ($hMLH1^-$) cells, embryon fibroblasts which were deficient in expression of the MLH1 protein failed to arrest at the G_2/M cell cyc checkpoint following 6-TG or IR. In contrast and as with HCT116 3-6 hMLH1-expressing cells, wild-ty embryonic fibroblasts derived from C57BL/6J mice which expressed normal levels of the MLH1 protein al demonstrated a dramatic G_2/M cell cycle checkpoint arrest in response to 6-TG or IR. Analyses of the S-pha cell populations in treated wild-type or MLH1 knock-out murine cells after 6-TG or IR demonstrated no appare S-phase cell population differences, indicating little variation in G_1 cell cycle arrest between MLH1-expressing ar MLH1-deficient cells after IR or 6-TG.

Summary of Data Supporting A Role of hMLH1 In G_2/M Cell Cycle Checkpoint Arrest After 5-Fluor 2'-deoxyuridine (FdUrd). We demonstrated above that DNA mismatch repair plays a role in the G_2/M ce cycle checkpoint using hMLH1-expressing or hMLH1-deficient HCT116 human colon carcinoma cells. We ha also demonstrated that DNA mismatch repair-deficient HCT116 cells were 6-fold more resistant to the cytotox effects of FdUrd and 2-fold more resistant to 5-fluorouracil compared to genetically matched, DNA mismatc repair-proficient HCT116 3-6 cells. HCT116 3-6 and HCT116 cells were treated with 30, 15, 4, 1, and 0.1 m FdUrd for 24 h and subsequently allowed to grow in drug-free medium. HCT116 3-6 cells treated with 1-4 m FdUrd showed a dramatic G_2/M cell cycle checkpoint delay and significant apoptosis (as detected by flo cytometry) as early as 12 h post-treatment. In contrast, HCT116 cells continued to grow without apparent ce cycle redistribution or apoptotic responses, exhibiting damage tolerance in a similar fashion as following 6-T treatment. Apoptotic responses characterized by flow cytometry were confirmed by Hoechst dye staining ar morphological examination by light microscopy. Interestingly, both HCT116 and HCT116 3-6 cells exhibited > fold increases in nuclear p53 protein levels after 1-4 mM FdUrd treatment, and the kinetics of their p53 inductic response appeared to be similar.

To determine if p53 protein responses or G_1 cell cycle checkpoint arrest played a role in this MMF dependent, FdUrd-induced G_2/M cell cycle checkpoint delay and apoptotic response, HCT116 and HCT116 3- cells were infected with retroviral vectors expressing the papillomavirus E6 gene. Infected and control cells we then treated with various doses of FdUrd as described above. Nearly identical cell cycle, survival and apoptot responses were observed in E6-expressing HCT116 3-6 cells compared to noninfected HCT116 3-6 cell indicating that p53 played no direct role in cell cycle, apoptotic or survival responses of these mismatch repai competent cells. Both E6-infected and noninfected HCT116 cells demonstrated near complete damage tolerance FdUrd, indicating that the lack of mismatch repair was crucial to responses to FdUrd in these cells and not the p5 responses or G_1 arrest, in general. Rescue from FdUrd cytotoxicity in HCT116 3-6 cells was observed via t administration (24 h posttreatment) with 10 μM thymidine, whereas 10 μM uridine had no effect on FdUr mediated G_2/M cell cycle arrest, apoptosis, or loss of survival in HCT116 3-6 cells. These data strongly suggest role for the MMR in the recognition of genetically unstable DNA, DNA mismatched bases caused by nucleotic pool imbalances via thymidylate synthetase inhibition, or by the rapid incorporation and removal of FdUrd in DNA.

C. RATIONALE AND/OR SIGNIFICANCE: Relationship to Mission of the DOE

Little or no molecular information regarding changes in gene expression after ultra-low doses of IR known in human or rodent cells. Changes in gene expression and alterations in the more subtle types of DN repair processes are undoubtedly important to the carcinogenic consequences of exposures to low doses of I ASRs in mammalian cells in response to low doses of IR have been reported for more than 40 years. Yet, t molecular basis for ASRs have been elusive. Over the last six years we identified, characterized, and isolate specific molecular probes (i.e., cyclin A, cdk2, xips5 & 13, cyclin D1 and PCNA) which are regulated during t establishment and challenging events of human cells demonstrating ASRs. The accomplishment of the propose goals outlined in this renewal application will allow us to elucidate the coupled responses of transcription, DN repair, and cell cycle progression which play a combined role in determining the extent of adaptive radioresistanc

by various human cells. The experiments outlined in the Specific Aims of Set #1 will allow us to establish th reagents with which we will be able monitor antisense and sense experiments and the influence of single genes (i.e xips5 & 13) on ASRs in human cells. We now know that the majority of genetic alterations which appear to affe whether or not ASRs occur in human cells, take place during low "priming" doses of IR. The experiments : Specific Aims #1-3 allowed us to investigate the role of xips5 & 13, cyclin A, PCNA, and cyclin D1 in ASRs : human or rodent cells. The use of controlled doxycycline-inducible vectors allowed us to regulate the overall exte of specific key regulatory proteins (by sense or antisense), and thereby possibly allow us to regulate ASRs. Thu the experiments proposed in this DOE grant renewal represents the first described molecular probes for ASF after low dose IR exposed cells.

In the second part of this grant we explored the role of a specific MMR protein (hMLH1) in the G_2/M ce cycle checkpoint arrest. Very little information is known about the mechanisms which regulate arrest at th important point in the cell cycle, although arrest at this checkpoint is clearly important for survival, apoptot responses, and carcinogenesis following IR. Our recent data (Preliminary data above) demonstrate a clear role f hMLH1 in a Mitotic checkpoint arrest following IR or FdUrd, using 6-TG as a control for the induction of MMI X-irradiation + FdUrd are the treatment of choice for colon cancer cells and the studies described in Specific Ain #4-7 have added clinical significance for treatment of non-HNPCC compared to HNPCC patients, which compris 5% of all colon cancer patients in the U.S. The experiments in Specific Aims #4-7 allowed us to further identif proteins which interact with hMLH1, and possibly act with hMLH1 to regulate G_2/M arrest.

Consortium/Contractual Arrangements. None.

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