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Abstract approved:

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Abstract

Genetic and environmental factors influence individual cancer risk. Mutations in DNA mismatch repair (MMR) genes increase colon cancer risk. Exposure to carcinogens, including the ubiquitous polycyclic aromatic hydrocarbons (PAHs), also is associated with increased cancer risk. To understand the combined effects of both environmental exposures and genetic risk, we investigated a role for MMR in cellular responses to PAHs. Benzo[a]pyrene (BaP) is a much-studied carcinogenic PAH; once metabolized to the diol epoxide (BPDE), it forms DNA adducts, which block DNA replication. This disruption signals for cell growth arrest, allows for adduct removal, and prevents accumulation of mutations. One of the first signaling events in cell growth arrest is the phosphorylation of the Chk1 kinase. To determine whether MMR deficiency influences BPDE-induced cell-cycle arrest, we measured increased phosphorylation of Chk1 in response to BPDE in MMR-proficient and -deficient human cell lines by immunoblotting. Preliminary data suggested sustained S-phase checkpoint in MMR-deficient cells relative to MMR-proficient cells. We also used flow cytometry to measure S-phase checkpoint activation. However, due to either technical or biological issues, or a combination, we could not find consistent patterns in cellular response to BPDE exposure in the two cell lines with either method.

Key Words: DNA Mismatch Repair, Polycyclic Aromatic Hydrocarbons, Cancer

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Investigating a Role for Mismatch Repair in Activating a Benzo[a]pyrene Diol Epoxide-Induced S-phase Checkpoint.

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I understand that my project will become part of the permanent collection of Oregon State University. My signature below authorizes release of my project to any reader upon request.

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Introduction

Cancer is a disease resulting in part from the accumulation of mutations in the somatic cells of multicellular organisms. While not every cell that accumulates mutations becomes cancerous, the statistics nonetheless speak of a problem that afflicts a significant portion of the population; lifetime cancer risk is one in three for women, and one in two for men. Its economic impact is enormous; the National Institute of Health (NIH) estimates that in 2007, cancer resulted in billions of dollars in direct medical costs and nearly 104 billion in indirect mortality costs due to loss of productivity (American Cancer Society 2012). Both the economic and emotional strain resulting from a cancer diagnosis can translate into a significant reduction of quality of life for anyone thus afflicted. In the United States, colorectal cancer is the third most commonly diagnosed cancer in both men and women but is the second overall cause of cancer death; in 2011, nearly 50,000 Americans died of colorectal cancer (American Cancer Society, 2012). Depending on the stage in which a colon tumor is detected, five-year survival rates for colon cancer may be as high as 74%, or as low as 6% (American Cancer Society, 2012). Thus early detection of tumors and precancerous lesions through regular screening is crucial to maximize the chances of survival and total remission. However, individual risk of developing colon cancer is decidedly nonuniform throughout the human population. Adequate screening precautions for cancer screening can differ considerably, based on family history, the individual's genetic makeup, and occupational or recreational exposure to known carcinogens. It is well established that both environmental and genetic factors play a role in shaping individual risk of developing cancer; however, how genetics and environment combine to determine personal cancer risk is not currently well understood. Thus a better understanding of the mechanisms by which genetic deficiencies affect cellular response to carcinogenic agents is essential to better calculate individual risk.

Genetic Factors Increasing Cancer Risk

Many genes have been identified as tumor suppressors or proto-oncogenes. These are genes which, when mutated such that the expressed gene shows phenotypic changes, can contribute to the development of cancer because of the disruption of vital cellular functions. Mutations for genes that are involved in critical pathways for genome maintenance can have a huge effect on individual ability to suppress deleterious mutations that can occur either spontaneously, or as a result of induction by carcinogenic agents. The resulting increase in mutation rate increases the likelihood of accumulating the necessary combination of tumor-suppressor and oncogene mutations required for carcinogenic transformation (i.e., tumor development).

One pathway commonly associated with familial colorectal cancers is the mismatch repair (MMR) pathway. This pathway is very highly conserved, and homologues of the different proteins exist in almost every known organism. One of its most important roles is the repair of errors that occur following of DNA replication, namely the correction of base mispairings and small insertions and deletions. This system recognizes deformation in the DNA structure resulting from mispaired DNA bases. The most important functional units in this pathway are MutLα (a heterodimeric protein formed from the proteins MLH1 and PMS2) and MutSα (a heterodimer of MSH2 and MSH6). In eukaryotes, MutLα is also essential for the strand discrimination function of MMR; it introduces nicks into the newly replicated DNA strand to assist with targeting correction to the error-containing strand (Crouse, 2010). Incorrectly inserted bases and DNA damage cause changes in the secondary structure of the DNA helix, to which the heterodimeric proteins MutSα and MutLα bind. They form a complex at the site of the incorrectly inserted base or lesion (Hsieh, 2001). The error-correcting activity of MMR, while undeniably important, is not the only way in which the pathway works to preserve genomic

stability. Additional functions have been identified in multiple studies, such as involvement in signaling apoptosis and cell cycle arrests in response to severe DNA damage by various genotoxic agents.

The loss of these genome-stabilizing functions results in individuals with genetically acquired mismatch repair (MMR) deficiency having a higher risk of developing cancer. Two genetically acquired MMR deficiencies have been identified and studied. Total (biallelic) genetic deficiency in one of the core MMR pathway proteins (MLH1, MSH2, PMS2 or MSH6), causes mismatch repair cancer syndrome (MMRCS). These individuals have a high risk of colorectal cancer, and also typically present with childhood lymphomas and leukemias, as well as a spectrum of medulloblastomas and glioblastomas (Ripperger, Beger, Rahner et al., 2009). Partial, or monoallelic, germline deficiency in one of the core MMR proteins causes Lynch syndrome, genetically inherited as an autosomal dominant condition. Lynch syndrome manifests most commonly as colorectal cancer, but increased incidence of cancers of the endometrium, ovary, and stomach have also been reported (Vasen, Moeslein, Alonso et al., 2007). While MMR heterozygotes may be functionally normal, deleterious mutations may occur in the second allele of the MMR gene that is already affected in a single cell. (Haggitt, Reid, 1986) (Kinzler, Vogelstein, 1996) This occurrence is known as loss of heterozygosity, and replication fidelity is compromised in these cells. As a result, accumulation of mutations tends to occur in the rogue cell, which may include additional genes coding for tumor suppressors and oncogenes. Consequently, Lynch syndrome individuals face a lifetime risk of developing cancer that may be as high as 85%. The mechanism by which MMR deficiency increases cancer risk following carcinogen exposure is not completely understood, but a reduced ability to suppress mutations induced by exposure to environmental carcinogens has been established in multiple studies. The recognition and correction of base mispairings and other types of DNA damage, while recognized as the main functions of the MMR pathway, appear to only be part of the way in which the pathway acts to preserve genomic stability.

Epithelial colon cells of MMR-deficient mice have displayed reduced capacity to activate the signaling for apoptosis compared to wild-type counterparts when exposed to BaP at high concentrations (Wu, Gu, Wang et al, 1999). Thus the indirect genome stabilizing effects of MMR, if ultimately cytotoxic to individual cells exposed to DNA-damaging chemicals, may help preserve the health of multicellular organisms by suppressing the development of tumors.

Environmental Factors Contributing to Colon Cancer

Exposure to certain environmental agents is known to be a risk factor that increases individual probability of developing cancer. Ionizing radiation, UV radiation and chemical carcinogens are all known to damage DNA with deleterious consequences. Ionizing radiation tends to induce oxidative lesions and double stranded DNA breaks; UV radiation causes a specific type of lesion known as a cyclobutane pyrimidine dimer (CPD), which creates a four-membered ring cross-linking two adjacent pyrimidines. Certain chemical carcinogens (such as aflatoxins, heterocyclic amines and polycyclic aromatic hydrocarbons (PAHs)) produce metabolites capable of adducting DNA, These adducts interfere with the process of DNA replication by forming a bulky lesion on DNA bases, making it impossible for the normal replicative DNA polymerase to elongate the strand past the site of the lesion. Of these chemicals, PAHs are by far the most abundant (Zhang, Yanxu, Shu Tao, 2007).

PAHs – Ubiquitous Environmental Carcinogens

PAHs are ubiquitous environmental pollutants that may be present either as the result of high temperature combustion of organic materials (Soclo, Garrigues, Ewald, 2000) or occurring naturally in heavy-fraction petroleum products such as coal-tar and diesel fuel. Because of their varied structure and

molecular weights, they also vary greatly in their physical and chemical properties, environmental persistence, and their effects on living systems. PAHs of higher molecular weight, particularly those with a structure composed of six or more benzene rings, are persistent pollutants and very resistant to environmental degradation. Their stability and chemical inertness make them capable of remaining in water or soil for extended periods of time, thus providing a significant bioaccumulation hazard and remediation challenge in heavily contaminated areas (Parrish, White, Isleyen et al., 2006). They can range from having very little biologic effect to being very potent mutagens, carcinogens and teratogens (Vanzella, Martinez, Colus, 2007). One of the best known and well-studied of the PAHs is benzo[a]pyrene (BaP), commonly detected in vegetable oils, grilled meats, tar, diesel exhaust and wildfire particulates (Kazerouni, Sinha, Hsu, et al., 2001), (Moret, Conte). The BaP carcinogenic activity serves as a standard for the tumor-inducing effects of PAHs, as the genotoxicity of the PAHs is often measured in terms of BaP equivalents. It rates near the top of the PAHs in terms of its ability to cause cancer in laboratory animals exposed to it; however, dibenzo[a,l] pyrene, one of the "fjord" region PAHs, has demonstrated significantly higher carcinogenicity relative to BaP.

The hydrophobic nature of BaP allows it to pass easily through cell membranes. Once inside the cell, BaP is bound by the aryl hydrocarbon receptor (Ahr), also known informally as the dioxin receptor. The Ahr is a cytosolic receptor with high affinity for cyclic aromatic compounds that, like PAHs, structurally resemble dioxins (Romagnolo, Degner, Selmin, 2010). When bound by a suitable ligand, the Ahr acts as a transcription factor for the xenobiotic response element (XRE), a regulatory region of DNA that affects the transcription of CYP1A1, epoxide hydrolase, and other metabolizing enzymes (Shimizu, Nakatsuru, Ichinose, 2002). BaP is metabolized into reactive intermediates, such as benzo[a]pyrene diol epoxide (BPDE), a highly reactive compound known to form adducts to DNA. The strongly electrophilic nature of BPDE makes it a prime target for nucleophilic attack by the amino

groups that are abundant on the purine bases of DNA. This reaction results in a covalent bond between the BPDE molecule and the purine, forming a bulky adduct to the DNA.

The BaP–derived adduct disrupts the replication of DNA by blocking the action of high-fidelity replicative polymerases such as polymerase delta (Polδ), which is largely responsible for the elongation of DNA. This disruption of synthesis results in two distinct cellular responses; one local, and one global in nature. The initial and local response to the DNA adduct is the local recruitment of translesion polymerases capable of bypassing the lesion, such as polymerase kappa (Pol κ). Polκ, while capable of inserting the correct base across form a BPDE-adducted purine, tends to be error-prone downstream from the site of the adduct by virtue of its larger, less specific active site and its lack of proofreading ability. This translates into an error rate several orders of magnitude higher than that of Polδ and other high-fidelity replicative polymerases (Bi, Barkley, Slater et al, 2006). This may result in untargeted mutations downstream from the site of the DNA adduct, recruiting the MMR proteins for their repair. Second, a global reduction in the rate of DNA synthesis, known as the S-phase checkpoint, may occur. The major effect of S-phase checkpoint is delaying further replication of the damaged DNA, as a result allowing more time for DNA repair. However, extensive DNA damage can cause global inhibition of firing at new origins of replication. In cases of severe damage to the DNA, the cell may signal for programmed cell death, or apoptosis. Initial signaling events in S-phase checkpoint include the phosphorylation of the protein kinase ATR, which in turn phosphorylates the protein kinase Chk1. The activation of these kinases is necessary for cell cycle regulation, and failure of these protective measures can put the cell at risk for accumulation of mutations, and, as a consequence, becoming cancerous.

The involvement of MMR in protection from accumulation of mutations following exposure to DNA-damaging agents is well established. Recent unpublished results from the Buermeyer laboratory have shown that MMR-deficient cells exposed to BPDE exhibited significantly increased BPDE-

induced mutations relative to their wild-type counterparts. In addition, published data demonstrates reduced apoptotic response in MMR-deficient cells exposed to PAHs (Wu, Gu, Wang, 1999). In addition, BPDE has been shown to induce the S-phase checkpoint in MMR-proficient cells (Guo, Faller, Vaziri, 2002), and MMR involvement in S-phase checkpoint activation induced by ionizing radiation (Brown, Ahbmy, Ravindra et al.,) and DNA-alkylating agents (Wang, Qin, 2003) has been reported. However, MMR involvement with a PAH-induced S-phase checkpoint specifically has not been previously addressed. We hypothesize that MMR has a role in signaling S-phase checkpoint when induced by BPDE exposure.

Overview of the Experiments

Genetic deficiencies in important regulatory pathways and carcinogenic chemicals are known to interact to determine an individual's risk of developing cancer. Several studies, including unpublished data from the Buermeyer laboratory, identify the MMR pathway as an important cellular response limiting genotoxic consequences of PAH exposure. Therefore we hypothesize that MMR is required for PAH-induced cell cycle arrest. Specifically addressed here is the involvement of MMR in S-phase checkpoint activation following BPDE exposure. To determine the impact of MMR status on activation of S-phase checkpoint in response to BPDE, we used two different approaches to measure S-phase checkpoint activation. For our experiment, we selected the MLH1-deficient cell line HCT116 and its subclone HCT116+ch3, with the MLH1 gene restored through the microsomal transfer of a third copy of human chromosome 3 lines (Koi, Umar, Chauhan et al, 1994). We produced lysates from cells exposed to BPDE that were used to create immunoblots later probed for PChk1. As an alternate method of determining differences in S-phase checkpoint activation between the two cell lines, we chose flow cytometry using propidium iodide staining as a method for quantifying DNA in the cells. Ultimately,

we were not able to detect consistent patterns in PChk1 accumulation or in cell cycle responses to BPDE for the cell lines. We experienced technical problems in both methods, but some inconsistencies in cellular responses to BPDE may have also occurred between different batches of the same cell lines. Some of the technical issues we experienced were eventually resolved, but others remained undefined and still cannot be explained. However, we have identified a number of technical changes that, if implemented, could improve the consistency of future experiments.

Materials and Methods

Cell Culture

HCT116 and HCT116+ch3 colorectal cancer lines (Koi, Umar, Chauhan et al, 1994) were obtained from Dr. T.A. Kunkel (NIEHS, Research Triangle Park, North Carolina). HeLa cells were from the Dr. S.K. Kolluri Laboratory, Oregon State University, Corvallis, Oregon). HCT116 and HCT116+ch3 cell lines were grown as attached cultures in complete Iscove's Modified Dulbecco's Medium (Thermo Fisher Scientific, Rockford, Illinois) containing 10% heat-inactivated fetal bovine serum (FBS) (Tissue Culture Biologicals, Long Beach, California) and 100 μg/mL penicillin-streptomycin (Cellgro, Manassas, Virginia) in 37° humidified incubators with 5% atmospheric CO_2. Similarly, HeLa cells were maintained in complete Dulbecco's Modified Eagle's Medium (Cellgro) containing 10% FBS (Tissue Culture Biologicals) and 100 μg/mL penicillin-streptomycin (Cellgro). Following initial thaw of frozen stocks, new cultures were maintained a minimum of one week prior to use in specific experiments. Cells were maintained subconfluently and passaged routinely to maintain optimal culture density. Cells were harvested using porcine trypsin to remove the cells from the tissue-culture plates, and cell counts were performed using a hemocytometer and trypan blue stain to distinguish viable from nonviable cells.

BPDE Treatment

Cells were plated in 145-mm tissue-culture plates containing complete medium. They were then allowed to recover from 24 to 48 hours prior to treatment. Following recovery period, the complete media was removed and replaced with serum-free medium. Stock solutions of BPDE (Midwest Research Institute, Kansas City, Missouri) in anhydrous DMSO were diluted to 100 to 500 μM; final exposure concentration ranged between 100 and 500 nM. Cells were treated either with the BPDE solution or an equal amount of DMSO for one hour. Following exposure, BPDE-containing medium was removed and replaced with complete medium after first rinsing the plates with 6.7 mM phosphate-buffered saline (PBS). Plates were then returned to the incubators for up to 48 hours prior to harvest and analysis.

Etoposide Exposure

HeLa cells were kept in culture a minimum of one week prior to exposure. Cells were harvested, counted and plated in 145-mm tissue-culture plates at approximately 1.5 million cells per plate. Following plating, we allowed them to recover in the incubator for 24 hours. We removed the complete medium and replaced it with complete DMEM medium to which we had added a 34 mM solution of etoposide dissolved in DMSO for a final etoposide concentration of 25 μM to ensure uniform dosing of plates. The plates were returned to the incubator overnight (18 hours) and removed for harvest and analysis.

Cell Harvesting and Lysate Preparation

Cells were harvested at various time points after exposure to BPDE. They were then washed in PBS and centrifuged. Following centrifugation, the pellets were re-suspended with 5 “pellet volumes”

of PBS (i.e, five times the pellet's volume estimated by visual comparison against known volumes of water in the same tube type), and then lysed using 5 "pellet volumes" of a 2x lysis buffer containing 100 mM Tris-HCl, 4% sodium dodecyl sulfate (SDS) and 20% glycerol to which a protease inhibitor cocktail tablet (Complete Mini EDTA-Free, Roche Diagnostics, Indianapolis, Indiana) had been added. Lysates were then boiled for five minutes at a temperature of at least 95° Celsius, snap-frozen in liquid nitrogen and stored at -80°.

To determine the protein concentration of the lysates, the colorometric Bicinchoninic Acid (BCA) Assay (Thermo Fisher Scientific, Rockford, Illinois) was performed as per manufacturer's directions. A standard curve was generated using bovine serum albumen (0 μg/mL to 2000 μg/mL). Protein absorbance at 538 nm was measured using a SpectraMax 250 (Molecular Devices, Sunnyvale, California) in conjunct with the program SoftMax Pro 3.1.1 (Molecular Devices, Sunnyvale, California). The protein concentrations of the lysates were then determined using interpolation based on linear regression of absorbance values for the standard curve, plotted using Prism 5.0d (Graph Pad Software, La Jolla, California).

Immunoblots

18-well pre-cast Criterion 4-12% Bis-Tris gradient gels and 7.5% Tris-HCl gels (Bio-Rad, Hercules, California) were used for separation of proteins via denaturing gel electrophoresis; running buffers were 1x XT MOPS (n-morpholino-propane-sulfonic acid) (Bio-Rad) and19.2 mM glycine and 2.5 mM Tris base (with 0.1% SDS per manufacturer's instructions. 25-80 μg of protein were loaded into each lane (depending on the specific experiment) and electrophoresed with 200 volts (constant voltage) for 50-55 minutes. Following electrophoresis, proteins were transferred to PVDF membrane (EMD Millipore, Billerica, Massachussetts) using the submerged transfer protocol at a constant voltage

of 100V for 30 minutes in a Criterion Blotter (Bio-Rad). Transfer buffer was 10% v/v methanol, 19.2 mM glycine and 2.5 mM Tris base in nanopure water. Following transfer, nonspecific antibody binding was suppressed using a blocking solution consisting of 25 mM Tris, 137 mM NaCl (sodium chloride), 27 mM KCl (potassium chloride), and 0.1% Tween 20 (TBST) with 5% weight/volume of either powdered dry milk or fraction five bovine serum albumen (Sigma-Aldrich, St. Louis, Missouri), either overnight at 4°C or 1 hour at room temperature (approximately 20°). Following blocking, membranes were probed with commercially available primary antibodies specific to the proteins of interest and according to manufacturer protocol for each antibody. Anti-PChk1 (P-Serine 345) rabbit polyclonal antibody (#2341S) was purchased from Cell Signaling Technology (Danvers, Massachusetts) and used at a concentration of 100-200 ng/mL; anti-Chk1 rabbit polyclonal antibody (sc-7898) was purchased from Santa Cruz Biotechnology (Santa Cruz, California). Mouse monoclonal anti-MSH6 antibody (Cat# 610918, BD Biosciences, San Jose, California) was used at a concentration of 100 ng/mL; mouse monoclonal anti-MLH1 antibody (NA28-100UG, Millipore, Cambridge, Massachusetts) was used at a concentration of 1 μg/mL. Mouse monoclonal anti-GAPDH antibody (CB1001, Calbiochem, Billerica, Massachusetts) was used at a concentration of 1 μg/mL. Following incubation with primary antibody, the blots were washed in TBST four times at intervals of 15 minutes for a total of one hour. The blots were then incubated with horseradish peroxidase-conjugated goat-anti-rabbit secondary antibody (#A0545, Sigma-Aldrich, St. Louis, Missouri) or goat-anti-mouse secondary antibody (31430, Thermo Fisher) at a concentration of 50 μg/mL or 10 μg/mL, respectively, and horseradish-peroxidase-conjugated modified streptavidin (5000x StrepTactin, Bio-Rad, Hercules, California) at 0.2 uL/mL for one hour at room temperature. Blots were then washed with TBST twice for 15 minutes, then twice for 15 minutes with TBS. Primary antibodies were visualized indirectly using Supersignal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific) as per manufacturer's instructions. Images

were captured using ChemiGenius Imager (Syngene, Frederick, Maryland) with the software program GeneSnap (Syngene). All image analysis was done using the program GeneTools (Syngene).

Flow Cytometry

Cells were harvested using trypsin and washed twice with PBS. The cells were then resuspended in PBS, then fixed by addition of 100% ethanol added dropwise to final concentration of 70% ethanol v/v. Fixed cells were stored at -20° for a minimum of 12 hours prior to staining with propidium iodide for analysis of DNA content. For staining, fixed cells were washed twice and resuspended in PBS, and suspended in a PBS solution containing 0.1% Triton (Dow Chemical, Midland, Michigan) to increase membrane permeability. The cells were then treated with 200 pg/mL RNAse and 20 ng/mL propidium iodide, and incubated at 37° for 30 minutes. Following incubation, the cellular suspension was filtered through a 37-micron mesh cloth to remove large aggregations of cells, and placed in a titer tube for cell-cycle analysis by the Cytomics FC 500 (Beckman Coulter, Brea, California). For analysis of the samples for DNA content, the flow cytometry analysis software programs MultiCycle and FlowJo VX 10.0.2 (Tree Star, Ashland, Oregon) were used. Cell-cycle modeling was done using the Dean-Jett-Fox algorithm, which fits G1 and G2 to Gaussian curves, and S-phase to a second-degree polynomial.

Results

Phenotypic Characterization of MLH1-proficient and –deficient Cell lines

Our goal was to determine differences in BPDE-induced S-phase checkpoint activation between MMR-proficient and –deficient cell lines. We used the previously characterized MLH1-deficient HCT116 and a subclone, HCT116+ch3, in which the MLH1-deficiency had been complemented by introduction of an extra human chromosome 3 expressing a wild-type MLH1 (Koi, Umar, Chauhan et al, 1994). The HCT116 cell line is derived from a human colon cancer and has biallelic mutations inactivating the endogenous MLH1 gene. These cell lines have been used in numerous published studies investigating the role for MLH1 in cell cycle, apoptotic and anti-mutagenic responses to genotoxic chemical exposures, including BPDE and other PAHs (Wu, Gu, Wang et al., 1999)

To verify that the HCT116 (MLH1-deficient) and HCT116+ch3 (MLH1-proficient) cell lines used in the BPDE exposures were phenotypically MLH1-proficient and –deficient (Koi, Umar Chauhan, 1994), we tested our HCT116 and HCT116+ch3 lysates for the presence of the essential MMR protein MLH1 via immunoblotting (Figure 1). As expected, HCT116 cells showed no discernible MLH1 signal. HCT116+ch3 cell lysates showed detectable MLH1 signal, verifying expression and accumulation of MLH1 in the cell line. To serve as a loading control and also to verify the accuracy of the BCA assay, the blot was then re-probed for the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using a monoclonal antibody specific to the protein. The blot showed similar accumulation of GAPDH in each lane, showing that the differences in protein accumulation in each were not a result of loading error or misquantification of protein concentration in the lysates. As an additional control, we probed the blot for the MMR protein MSH6. Per our expectations, both cell lines showed visible MSH6 signal. Thus we were satisfied of the MMR status of our cell lines. An unexpected result was that the

HCT116 cell lines showed visibly weaker MSH6 signal than the HCT116+ch3 (Figure 1). This result was consistent across the blot and has been reproduced in subsequent blots as well.

GAPDH as a Loading Control

Standard practice for immunoblotting is to probe for the presence of a "housekeeping" protein, expected to be present at similar levels in cells, regardless of treatment. In initial experiments, we used GAPDH and detected similar band intensities in different extracts following different cellular treatments. However, one concern is the extent of the linear dynamic range for the chemiluminescent signal (defined as a linear increase in measured signal intensity with increased protein loaded) with a highly expressed protein like GAPDH. With excessive protein amounts loaded onto the gel, chemiluminescent signal intensities typically plateau, preventing the detection of lane-to-lane differences in protein load. Therefore, to identify the linear dynamic range for GAPDH signal, we analyzed immunoblots consisting of a standard curve prepared from a single whole cell lysate in load amounts varying from 20-60 μg protein. We selected two different antibody concentrations of 1.25 μg/mL (not shown) and 625 ng/mL (Figure 2) in order to optimize the signal development. Results demonstrated increasing GAPDH signal intensities up to approximately 30 μg of whole cell lysate loaded (Figure 2, lanes 1-3 and accompanying graph). No additional consistent increase in signal was measured with 35-60 μg of protein analyzed (Figure 2, lanes 4-7 and accompanying graph). These results suggest that detection of linear increases in GAPDH chemiluninescent signal requires whole cell protein loads of less than 30 μg. More work, specifically additional immunoblots loaded with 20 μg or less of protein, would be needed to identify the full linear dynamic range for GAPDH signal.

Quantification of PChk1 Accumulation by Immunoblot Analysis

To investigate potential differences in the activation of S-phase checkpoint, a known cellular response to BPDE exposure, in MMR-proficient versus –deficient cells, we measured the relative accumulation of Chk1 kinase phosphorylated on serine residue 345 (PChk1) in cells treated with BPDE. In an initial experiment (Figure 3), we observed visible signal that co-migrated with proteins at a molecular weight of 57 kDa, the molecular weight of PChk1. This putative PChk1 signal was similar for all DMSO-treated control lysates (lanes 1-4), showing no significant difference among the different time points and cell lines. In contrast, the level of PChk1 appeared increased in a dose-dependent manner (i.e., increasing with BPDE concentration) 24 hours following exposure (compare lanes 5 and 6 and 9 and 10 to lanes 1 and 2 and 3 and 4, respectively). Differences in PChk1 accumulation between the cell lines were discernible at the 48-hour time point. In the MMR-proficient cell lines (lane 12), the PChk1 concentration returned to baseline levels. However, the MMR-deficient HCT116 cell line (lane 11) showed sustained accumulation of elevated levels of PChk1. These preliminary results were not consistent with our initial hypothesis that MLH1-deficient cells would show reduced activation of the S-phase checkpoint in response to BPDE. Instead, it appeared that MLH1-deficient cells showed a similar ability to activate the checkpoint (as measured by accumulation of PChk1). In addition, continued accumulation of PChk1 in MLH1-deficient cells 48 hours following exposure suggested that the checkpoint remained active for longer than in MLH1-proficient cells.

Detection of a specific PChk1signal in some of our lysates was complicated by an additional nonspecific band, migrating slightly more slowly than the putative PChk1 signal. This nonspecific signal provided significant interference with the detection of PChk1. (See, for example, the analysis of HeLa

cell lysates in Figure 5 below.) Thus, we sought to find a gel with a different protein migration pattern than the 4-12% Bis-Tris gradient gels that we had been using for electrophoresis. Specifically, we hoped to find a gel that would provide better protein separation in the range between 37-75 kDa, thus distancing the two bands from each other. Based on protein migration pattern literature published by Bio-Rad, we selected the 18-well 7.5% Tris HCl gel for electrophoresis. Using previously analyzed samples (see Figure 2) of protein lysates of BPDE-exposed HCT116 and HCT116+ch3 cells, we probed the resulting immunoblot for PChk1. The analysis showed a lack of nonspecific banding in the area of concern (the region between 50 and 75 kDa) and a clear, well-resolved band migrating at approximately 57 kDa (Figure 4). As before (Figure 2), the apparent PChk1 signal was increased in lysates of BPDE-exposed cells harvested 24 hours after exposure, irrespective of MLH1 status (Figure 4, compare lanes 5 and 6 to lanes 1 and 2). In addition, at 48 hours after exposure, PChk1 signal associated with the MMR-proficient HCT116 +ch3 cell lysate showed a return to baseline levels (lane 8), whereas the PChk1 signal remained elevated in the lysate of the MMR-deficient HCT116 cells (lane 7) .

Positive Control for PChk1

To determine whether signal detected at approximately 57 kDa represented PChk1, we sought to identify a positive control for PChk1 that we could include on our immunoblots to verify the identity of the signal in the lysates as PChk1. HeLa cells treated for eighteen hours with the chemotherapeutic drug etoposide are reported to show a strong activation of S-phase checkpoint with PChk1 accumulation. We obtained HeLa cells from the Kolluri Laboratory (Oregon State University) and exposed a growing culture of cells to 25 μM etoposide, following the recommendations of technical support at Cell Signaling Technology. Eighteen hours after exposure, the cells were harvested and whole cell lysates were prepared. Protein lysates were separated via gel electrophoresis, and the blot was then probed for

PChk1 (Ser 345). This experiment was repeated three times. Unfortunately, the results of these control experiments were inconsistent. Although, we did detect weak signal that may represent PChk1, and that was apparently increased over the signal in control lysates (Figure 5, compare lanes 2, 6 and 10 with lanes 1, 5 and 9, respectively), in other experiments no difference in PChk1 accumulation was detected between the DMSO-treated lysates and the etoposide-treated lysates (Figure 5, compare lanes 4, 8 and 12 with 3, 7 and 11, respectively). Because PChk1 signal had been difficult to detect in the HCT116 and HCT116+ch3 lysates at the recommended concentration of 100 ng/mL in blocking solution, we also tried increasing the concentration of antibody to improve signal intensity. Based on a comparison of three different concentrations (400 ng/mL, 200 ng/mL and 100 ng/mL) of PChk1 primary antibody used to probe lysates loaded in parallel, it appeared that visibly improved signal intensity was achieved at concentrations of 400 ng/mL (Figure 5, compare lane 10 with lanes 6 and 2).

Analysis of a time course for activation of the S-phase checkpoint in response to BPDE

Published data from the Vaziri Laboratory (Bi, Vaziri, 2002) demonstrated strong activation of the S-phase checkpoint with maximal accumulation of PChk1 above baseline between 1-4 hours following BPDE exposure. Therefore, to identify any potential differences in the timing of the initial activation of the S-phase checkpoint in MLH1-proficient and –deficient cells, we attempted to analyze samples of HCT116 and HCT116+ch3 cells harvested at 1, 4, 6 and 24 hours following BPDE exposure. As an additional control, we included analysis of an additional MMR-proficient cell line (HeLa). Unfortunately, we experienced technical difficulties with these analyses, primarily associated with inconsistent detection of, and low signal intensities associated with, the PChk1 signal (Figure 6 and data not shown). To circumvent these difficulties, we increased the concentration of BPDE to 500 nM and chose a gel with narrower lanes to improve signal detection and quantification by concentrating the

protein in a smaller area and giving a smaller, more intense signal (Figure 6). We selected a 26-well (15 μL well capacity) 7.5% Tris-HCl gel for electrophoresis. However, following immunoblot analysis, the putative PChk1 bands still were weak and difficult to quantify. There was no consistent measurable increase in signal associated with BPDE treatment in any of the cell lines at any of the time points analyzed. We were unable to determine whether the difficulties here with detection of PChk1 signal were due to technical issues associated with the immunoblot procedure, or due to biological differences in cellular responses versus our initial experiment.

Cell Cycle Analysis

As an alternative method for determining whether MMR-proficient and -deficient cells differed in S-phase checkpoint activation following exposure to BPDE, we used flow cytometry to analyze progression through the cell growth cycle. In this technique, the fluorescent dye propidium iodide (PI) is used to stain the DNA. PI, an intercalating agent, incorporates itself into the DNA helix at intervals of approximately 4-5 bases. The DNA content for each cell can therefore be estimated from the intensity of the red fluorescence emitted by the PI and detected using the flow cytometer. Analysis software is used to plot the number of cells (y-axis) against the intensity of PI fluorescence (x-axis). The peak containing the lowest intensity of fluorescence (and therefore 2n DNA) is set as G1, and identifies cells that have not yet commenced DNA replication. The second peak, normally identified as G2, is usually smaller, and is located twice as far from the origin as the G1 peak, identifying cells that contain 4n DNA and have finished replication. Cells falling between the two peaks contain an intermediate amount of DNA, indicating that the cells have started, but not completed, DNA synthesis, and are therefore considered to be in S-phase. Using modeling systems such as the Dean-Jett-Fox algorithm, which fits G1 and G2 to Gaussian curves and S-phase to a second degree polynomial, the percentage of cells in

each phase can be estimated. Normally, for untreated cells, it is expected that a majority of cells are in G1, then G2, with the remainder in S-phase (Hardman, Afshari, Barrett, 2001). Activation of the S-phase cell-cycle checkpoint results in an increase in the number of cells in S-phase, which can be identified as an increase in the percentage of cells with an intermediate DNA content measured by PI-fluorescence.

To identify changes in cell-cycle phase distribution following BPDE exposure of MLH1-proficient and –deficient cells, we treated HCT116+ch3 and HCT116 cells to 500 nM BPDE or DMSO and harvested them for analysis of DNA content at various time points following exposure (Figure 7). The resulting histograms were analyzed using visual inspection and software modeling of the area under the curves. Unfortunately, we did not identify a significant increase in the percentage of S-phase cells at any time point following BPDE exposure with either cell line. Thus, we were not able to detect any significant and reproducible difference in cellular response to BPDE between the two cell lines. Although control DMSO-treated cell populations generally appeared normal by visual inspection, the quantitative analysis using modeling software (two different programs) was not successful. Modeled populations either appeared to dramatically underestimate the percentage of cells in G2/M, and therefore overestimate the percentage of cells in S-phase, or were unable to fit the data successfully. The difficulty appeared to be a result of many of the G1 and G2 peaks having “shoulders” that interfered with curve fit using the software analysis. For example, one quantitative analysis of HCT116+ch3 cells identified the majority of the cells in S-phase (65-80%) with approximately 20% in G1 and 0-2% in G2 (Figure 7a). This made it difficult to determine if the BPDE-treated HCT116+ch3 cells (Figure 7a, lower panels) showed a shift to an increased percentage of cells in S-phase at the 4- and 6-hour time points. There was an apparent increase in the percentage of G2/M cells at 24 hours, possibly suggested recovery from an S-phase checkpoint; however, given the difficulty in quantitatively analyzing the data, we could not be certain

that any apparent shifts were due to the treatment received by the cells. We had anticipated that the MMR-deficient cells (Figure 6b) would show a different cycle pattern than their MMR-proficient counterparts, possibly showing, sustained checkpoint activation, consistent with our preliminary PChk1 immunoblotting findings . Instead, we were unable to determine any obvious increase in percentage of cells in S-phase in either cell line following BPDE exposure. Because of the difficulty in fitting the histograms of many of the samples to the algorithm, we were not able to evaluate meaningful quantitative differences in cell cycle behavior between the different samples.

Discussion

We attempted to determine the impact of MMR status on activation of a PAH-induced S-phase checkpoint using flow cytometry and semiquantitative protein immunoblotting. Previous studies had shown that PAHs induce S-phase checkpoint (Guo, Faller, Vaziri, 2002) and that the MMR pathway is necessary for S-phase checkpoint activation in response to ionizing radiation and high-dose alkylating agents (Brown, Rathy, Kamath, 2003). We had expected that, by use of flow cytometry and protein immunoblotting, we would be able to detect difference in behavior between our two cell lines. Preliminary results, i.e., the product of immunoblots from lysates produced frome one BPDE exposure experiment, suggested that our hypothesis was false. PChk1 accumulation levels, one of our measures of S-phase checkpoint activation, appeared to be approximately equal in both MMR-proficient and –deficient cell lines 24 hours after expoiure, with levels that were elevated relative to DMSO controls. A return to baseline PChk1 at 48 hours in the MMR-proficient cell lines, also reproduced in the same lysate, may have been the result of misquantification of the protein concentration of the lysate, but could have also suggested resolution of the S-phase checkpoint induced by BPDE. These results were not observed in subsequent experiments, as technical difficulties in some of the immunoblots made quantification of PChk1 difficult. No differences in S-phase checkpoint activation were observed via flow cytometry. Cellular response of the BPDE-treated cells was difficult to determine because of abnormal cell-cycle behavior in the control samples. We were ultimately unable to determine if these inconsistent results were a result of biological differences between individual cultures of the same cell lines, technical issues, or some combination of both. Some of the technical issues were eventually corrected, but others evaded resolution. We do, however, believe that the question is still worth addressing, as it could help shed light on the mechanisms by which active MMR helps to suppress PAH-induced mutations, and therefore may help explain development of cancer in individuals with MMR

deficiency. There are a number of steps that we have identified that could improve the possibility of determining what differences, if any, exist between MMR-proficient and deficient cells in activating a PAH-induced S-phase checkpoint.

Acquiring Cell Cultures From the Vaziri Laboratory

In a previous study, H1299 cells showed remarkable cellular response when exposed to BPDE (Guo, Faller, Vaziri, 2002). We were not able to reproduce this result with respect to PChk1 accumulation and S-phase checkpoint activation using HCT116 and HCT116+ch3 cell lines. A potential solution for this problem would be to obtain H1299 cells from the Vaziri Laboratory, that have not been propagated since the original experiment. This would improve the likelihood of reproducing Dr. Vaziri's results by obtaining cells that are more phenotypically similar to the cells in the original experiment. If we succeeded in replicating Dr. Vaziri's results with the H1299 cell lines, we could anticipate returning to our original question regarding S-phase checkpoint and MMR status. H1299 cells are TP53-deficient but MMR-proficient; with the use of siRNAs, they could be rendered temporarily deficient in the MMR protein of our choice.

Using siRNA to Produce MMR-deficient Cell Lines

For our MMR-proficient cell line, we have been using the HCT116+ch3 line, which consists of the HCT116 line to which a third copy of human chromosome 3 carrying a wild-type MLH1 gene has been transferred, along with a neomycin resistance gene as a selection agent. The use of HCT116+ch3 cell lines as a positive control in an experiment testing the ability of cells to activate S-phase checkpoint is somewhat problematic. Microsomal transfer of human chromosomes can result in over-expression of

additional proteins that may affect the outcome of the experiment in unforeseen ways. While differences between the HCT116 and HCT116+ch3 cell lines may be attributable to the differences in MMR status, there are many other genes besides MLH1 on the transferred chromosome that could affect the experiment. In particular, the gene for ATR, a checkpoint kinase that is known to be upstream from Chk1 in the S-phase arrest signaling cascade, is located on chromosome 3. Thus the transfer of a third copy of chromosome 3 into the HCT116+ch3 cell line could cause the over-expression of the kinase ATR. Over-expression of ATR could result in differences in accumulation patterns of PChk1 in the two cell lines that are unrelated to MMR status. They may instead be due to the presence of an extra copy of the ATR gene, which is directly responsible for Chk1 phosphorylation.

Transient knockdown of selected MMR proteins for the purposes of exposure would be preferable for a number of reasons. First, we can be certain that the only difference between the two cell lines is that one has been transfected with siRNA for the gene of interest. The transfection process can be toxic to cells and may result in increased mortality in transfected cells. There are, however, alternate reagents and a number of controls that could be used to account for the cytotoxicity, such as transfection with silent siRNAs, which would subject control cells to the same treatment as the MMR protein knockdown cells.

Another reason that the transient knockdown method is preferable is that partial MMR deficiency increases the mutation rate in cells. Thus, cells continually acquire new mutations, some of which may cause them acquire properties that allow for the development of malignancy. When working with these cell lines, it therefore becomes difficult to ascertain that differences in cell-cycle behavior and PChk1 accumulation are due to MMR status alone in the HCT116 cell line, which was excised from a human colon tumor. However, siRNA can be used to create a MMR-deficient cell culture from any cell line, even from transformed but noncancerous cells. Transient knock-down is much less likely than a

permanent MMR-deficiency to result in the accumulation of mutations that could indirectly affect the outcome of the experiment. The transfected cells can then be used for the BPDE exposure and harvested for analysis by method of choice.

Using Synchronous Cultures

The use of synchronous cultures would possibly help improve the sensitivity of flow cytometry as an assay of DNA content and S-phase checkpoint activation (Schorl, Sedivy, 2006). Based on previous studies (Guo, Faller, Vaziri, 2002), we expected to see an increase in the number of cells in S-phase following BPDE exposure, especially at the earlier time points. However, we saw no clear pattern in the treated cells. One possibility is that too few cells were in S-phase at any given time and the change in the number of cells in S-phase arrest was too small to see. This problem could be addressed using synchronous cultures at the time of exposure, because all the cells in the population would be in the same phase at the same time. There are a number of methods that could be used to synchronize cell cultures which have varying degrees of disruptiveness to the cell cycle. As the intent of the research is to observe the cell cycle–disrupting effects of BPDE alone, the use of antimitotic chemicals such as colchicine and aphidicolin would best be avoided. Instead, it would be preferable to use a technique that separates cells based on the phase that they are currently experiencing. The simplest, least invasive method would be the mitotic shake-off. This technique involves growing adherent cells in standard tissue culture flasks and taking advantage of the tendency of many cell types to round up as they go through mitosis. It is usually enough to shake the flask and rinse with media to dislodge the mitotic cells which can then be collected and cultured (Schorl, Sedivy, 2006). Similarly, the process of centrifugal elutriation is also less disruptive than cell cycle-disrupting chemicals (Bafalvi, 2008). Through the use of a centrifuge with a specially designed rotor and a low density elutriating

buffer, cell populations that are uniform in size may be divided into groups by phase, as weight increases with DNA content as the cycle progresses towards G2. This method is likely to yield a higher quantity of cells available for culture than the mitotic shake-off, as only a small percentage of cells within a culture are undergoing mitosis at any given time. In one study, the first fraction of elutriation from a population of cycling mammalian cells contained more than 90% cells in G1 (Edelbrock, Kaliyaperumal, Williams, 2009). One potential problem with elutriation is that if there is a great deal of variation in size and weight among the cells in a population, they will not necessarily be sorted by phase. This problem, however, may be minimized if the cell cultures, whichever line they may be, are subcultured prior to elutriation, which should result in greater genetic and phenotypic uniformity of the population so they can be better sorted.

Working with cell cultures that are uniform could make the process of elutriation more efficient in that cultures where the cells vary greatly in size and weight are difficult to sort by phase. Having uniform cultures would improve the sensitivity of flow cytometry in measuring differences in S-phase checkpoint between cell populations following BPDE exposure. It is also possible that having more uniform cell lines would improve signal intensity of S-phase checkpoint protein markers in protein immunoblots, because genotypically uniform cells would be more likely to have the same response (i.e., phosphorylation of Chk1) to a carcinogen. HCT116 and HCT116+ch3 are cell lines that originated from mutator phenotype cells excised from a colon tumor. It is thus to be expected that these cells would experience a higher than normal mutation rate. Thus, with time, the cultures may become genetically heterogeneous and eventually display multiple polymorphisms in different genes that could generate a distinctly nonuniform response to a mutagenic stimulus. Producing subcultures from individual cells of existing HCT116 and HCT116+hCh3 cell lines would help isolate more homogeneous cultures. More uniform cellular response in cell populations could improve the sensitivity of flow cytometry as an assay

for S-phase checkpoint activation. Though we do not ultimately know the reason for the inconsistency in PChk1 signal in our immunoblots, it is also possible that through the process of subculture and synchronization, we might hope to see definitive PChk1 signal in the lysates that follows distinct patterns with respect to MMR status, dose and time following BPDE exposure.

PChk1 as a Positive Control

One major difficulty of the experiment was our inability to verify the identity of the 57 kDa signal in our protein immunoblots as PChk1. Despite several attempts to create a positive control from HeLa treated with 25 μM etoposide, we were unable to consistently detect strong PChk1 signal in our immunoblots from these lysates, and were unable to determine if the source of the inconsistency was the antibody or inconsistent accumulation of PChk1. Another positive control recommended by Cell Signaling Technology was a lysate prepared from HeLa cells irradiated with UV radiation at 20 $J \cdot m^{-2}$. However, were we to attempt this experiment, we would run the risk of obtaining similarly inconsistent results using another biological control such as a treated cell line, not knowing whether it was a failure of the antibody or a failure of the cells to accumulate PChk1. A more certain, and much simpler approach would be to obtain PChk1. While PChk1 may be difficult to acquire as a purified protein, purified Chk1 may be obtained and phosphorylated in vitro. The PChk1 could then be added to some of our cell lysates for a positive control. Using this would allow us to observe how the protein migrates in conjunction with native proteins that are present in the lysates and perhaps make quantitation of PChk1 possible. Additionally, failure of the antibody to consistently detect purified PChk1 (Ser 345) would show definitively that the antibody was not sensitive to the protein, requiring a new PChk1 antibody to be produced in the laboratory, or purchased.

Summary

Ultimately, we were unsuccessful at determining the differences in BPDE-induced S-phase checkpoint activation between MMR-proficient and –deficient cell lines. Our preliminary results, though by no means definitive, suggested that MMR-deficient cell lines possess the same capability to activate S-phase checkpoint in response to BPDE exposure, at least as measured by PChk1 accumulation. Cell-cycle analysis, unfortunately, failed to yield useful results. However, we have identified a number of ways that this experiment could be improved. If successful, this research could help direct future research so that the involvement of the MMR system in S-phase checkpoint activation is better understood. This could help elucidate the biochemical mechanisms that cause Lynch syndrome individuals to have a higher risk of developing cancer when exposed to PAHs. Understanding these mechanisms could lead to a better comprehension of how the effects of genetic deficiencies and environmental carcinogens combine to determine an individual's risk of developing cancer. Such insight could lead to advances in intervention efforts for vulnerable individuals such as earlier and more frequent cancer screening, drug prophylaxis and identification of occupational and recreational factors that may serve to increase cancer risk.

Figures

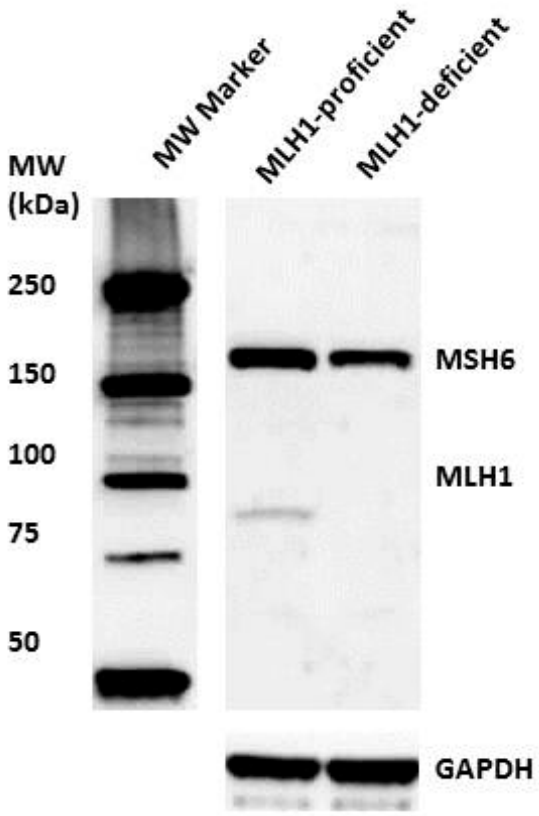

Figure 1. MLH1 accumulation in MLH1-proficient and –deficient cells. The cellular abundance of MLH1 and MSH6 was analyzed by semi-quantitative protein immunoblotting using whole cell lysates of HCT116+ch3 (MLH1-proficient) and HCT116 (MLH1-deficient) cells. Detection of GAPDH (probed separately) served as a loading control. An analysis of representative lysates is shown, and the position of each protein band analyzed and the molecular weight standards are labeled.

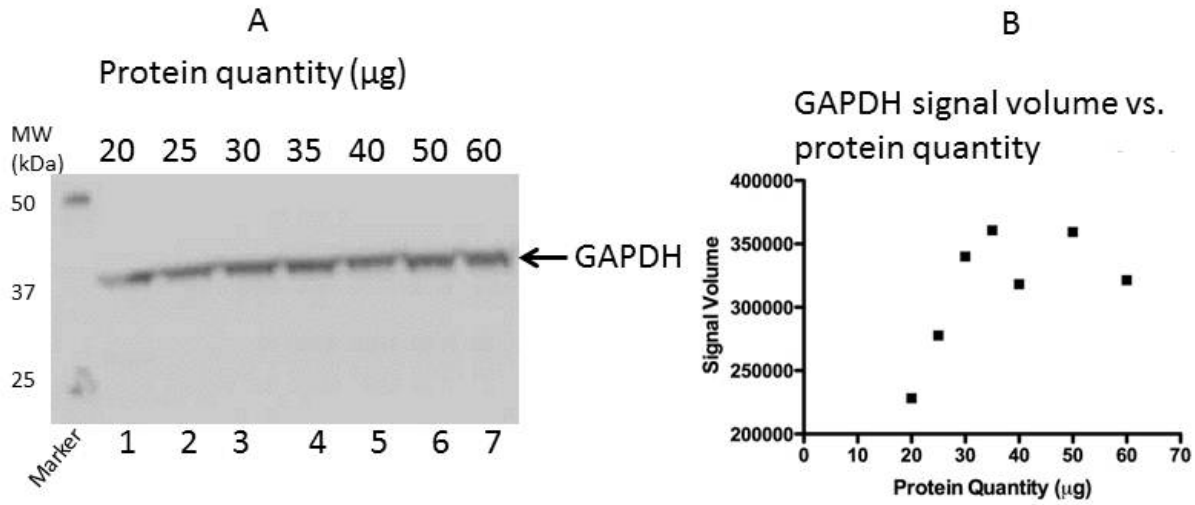

Figure 2. GAPDH standard curve. The upper limit of the linear dynamic range for GAPDH chemilumunescent signal was determined by semi-quantitative immunoblotting. Whole cell lysates of HCT116+ch3 cells (20-60 µg as indicated) were analyzed using a 4-12% Bis-Tris gel (panel A), with the measured GAPDH signal intensities plotted as a function of protein loaded (panel B).

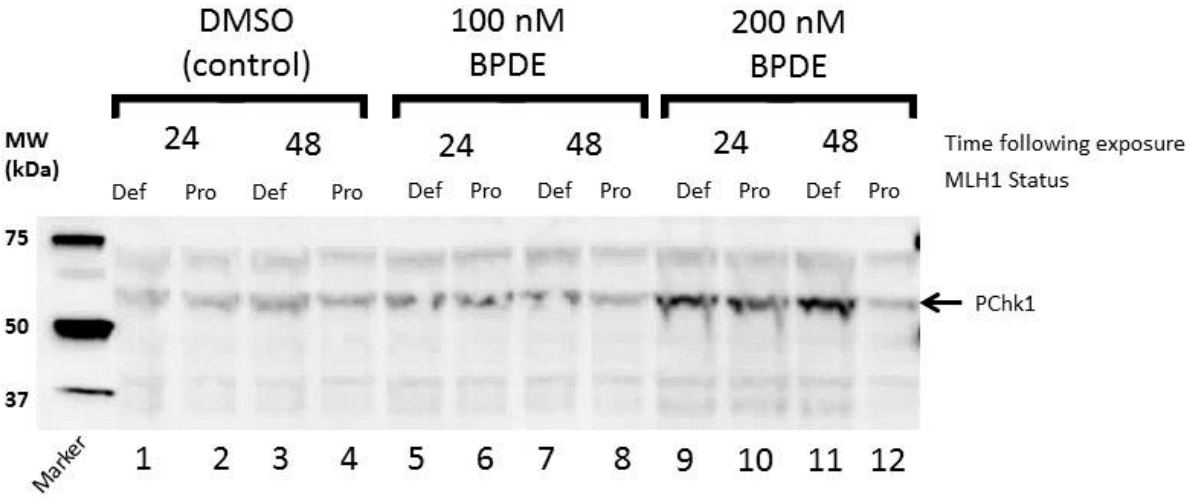

Figure 3. Accumulation of PChk1 in MLH1-proficient and –deficient cells exposed to BPDE. The cellular abundance of Chk1 phosphorylated on serine 345 (PChk1) was determined by semi-quantitative protein immunoblotting of whole cell lysates prepared from cells exposed for 1h to BPDE (100 or 200 nM as indicated, lanes 5-12) or DMSO solvent control (lanes 1-4). Lysates were prepared after the indicated time periods. Labels identify the lysates prepared from MLH1-proficient HCT116+ch3 (Pro) and MLH1-deficient HCT116 (Def) cells, the position of the putative PChk1 signal and the molecular weight standards used

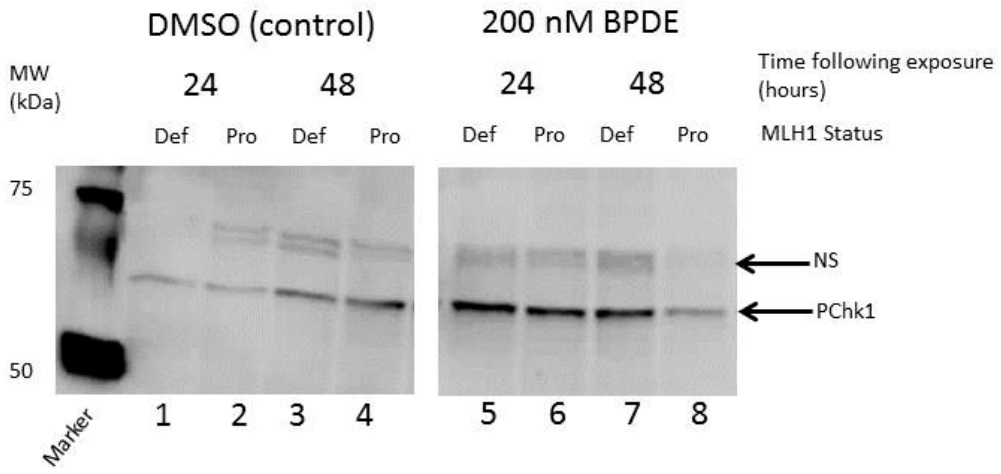

Figure 4. Repeat analysis of PChk1 accumulation in MLH1-proficient and –deficient cells exposed to BPDE. The cellular abundance of PChk1 was determined by immunoblotting as described in Figure 3 using a 7.5% Bis-Tris denaturing protein gel for electrophoresis. Samples analyzed are a subset of the same samples analyzed in Figure 3; labels follow the same scheme and include identification of the nonspecific band described in the text.

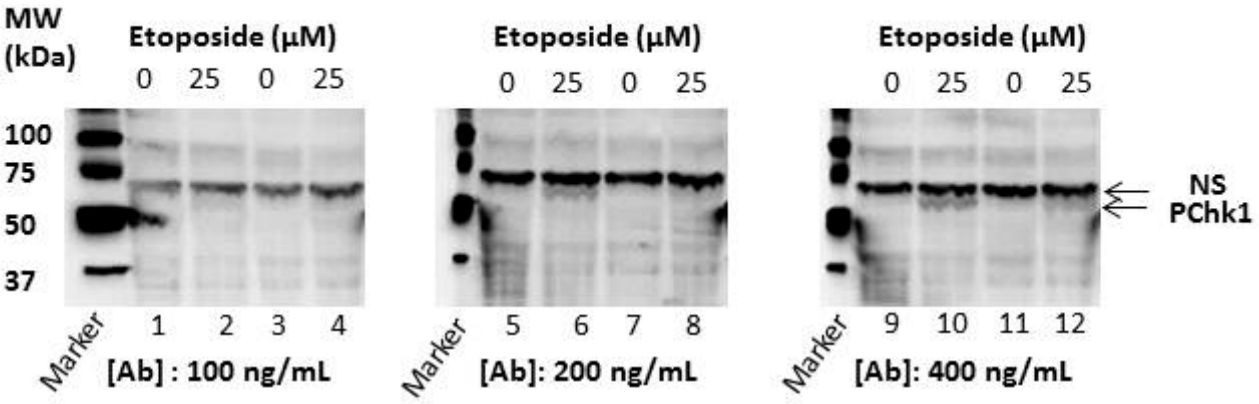

Figure 5. Accumulation of PChk1 in etoposide-treated HeLa cells. The cellular abundance of Chk1 phosphorylated on serine 345 (PChk1) was) was determined by semi-quantitative protein immunoblotting of whole cell lysates prepared from cells exposed to 25 μM etoposide (25; lanes 2, 4, 6, 8, 10 and 12) or DMSO solvent control (lanes 1, 3, 5, 7, 9, 11). Lysates were prepared 18 hours after exposure. Labels identify the position of the putative PChk1 signal, nonspecific signal (NS) and the molecular weight standards used

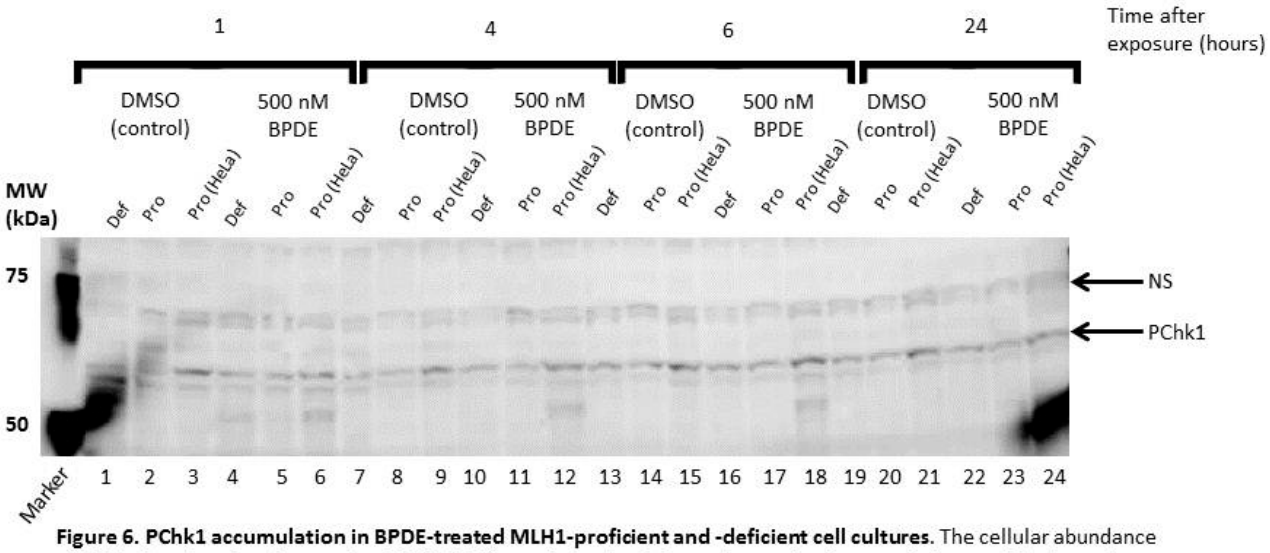

Figure 6. PChk1 accumulation in BPDE-treated MLH1-proficient and -deficient cell cultures. The cellular abundance of Chk1 phosphorylated on serine 345 (PChk1) was determined by semi-quantitative protein immunoblotting of whole cell lysates prepared from cells exposed for 1h to BPDE (500nM as indicated, lanes 4-6, 10-12, 16-18, and 22-24)) or DMSO solvent control (lanes 1-3, 7-9, 13-15, and 19-21). Lysates were prepared after the indicated time periods and electrophoresed on a 26-well 7.5% Tris HCl gel. Labels identify the lysates prepared from MLH1-proficient (HCT116+ch3 (Pro) and HeLa (Pro (HeLa)) cells) and MLH1-deficient HCT116 (Def) cells, the position of the putative PChk1 signal and the molecular weight standards used.

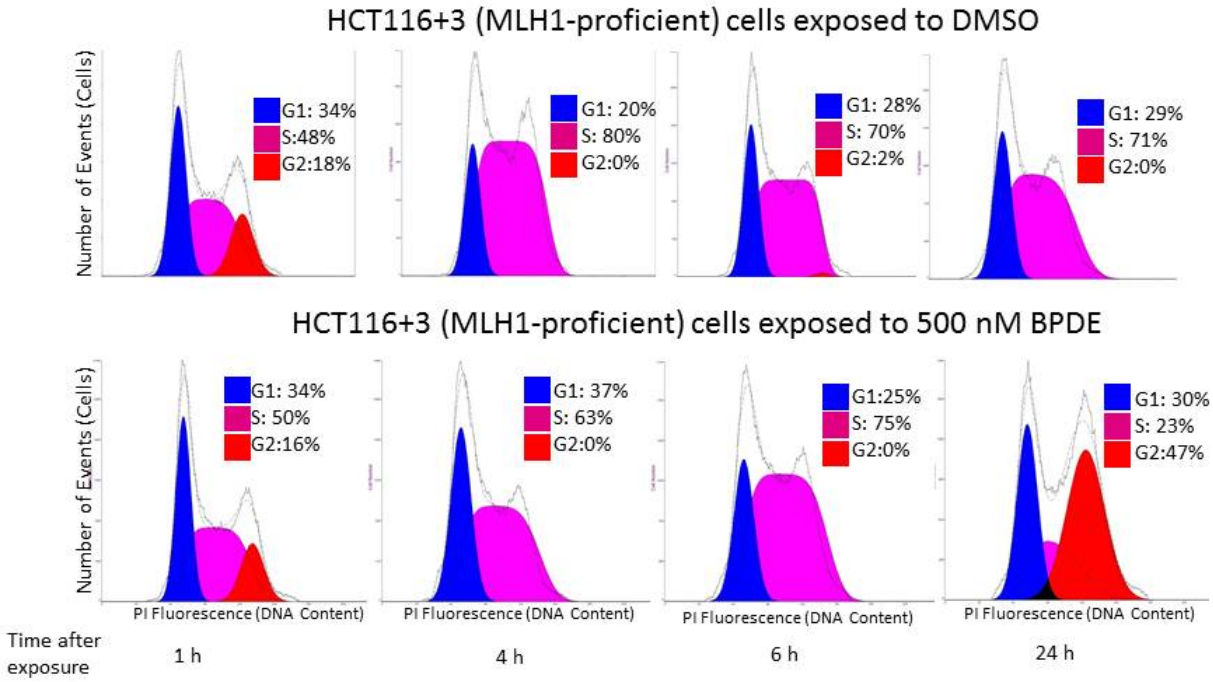

Figure 7a. Cell-cycle analysis of DMSO- and BPDE-exposed HCT116+3 cells. DNA content of HCT116+3 (MLH1-proficient) cells was measured via flow cytometry. HCT116+3 (MLH1-proficient) cells were exposed to 500 nM BPDE or DMSO for 1 hour. Cells were harvested at the indicated time periods following exposure. Labels indicate the percentage of the cell populations in each of the phases G1, S and G2.

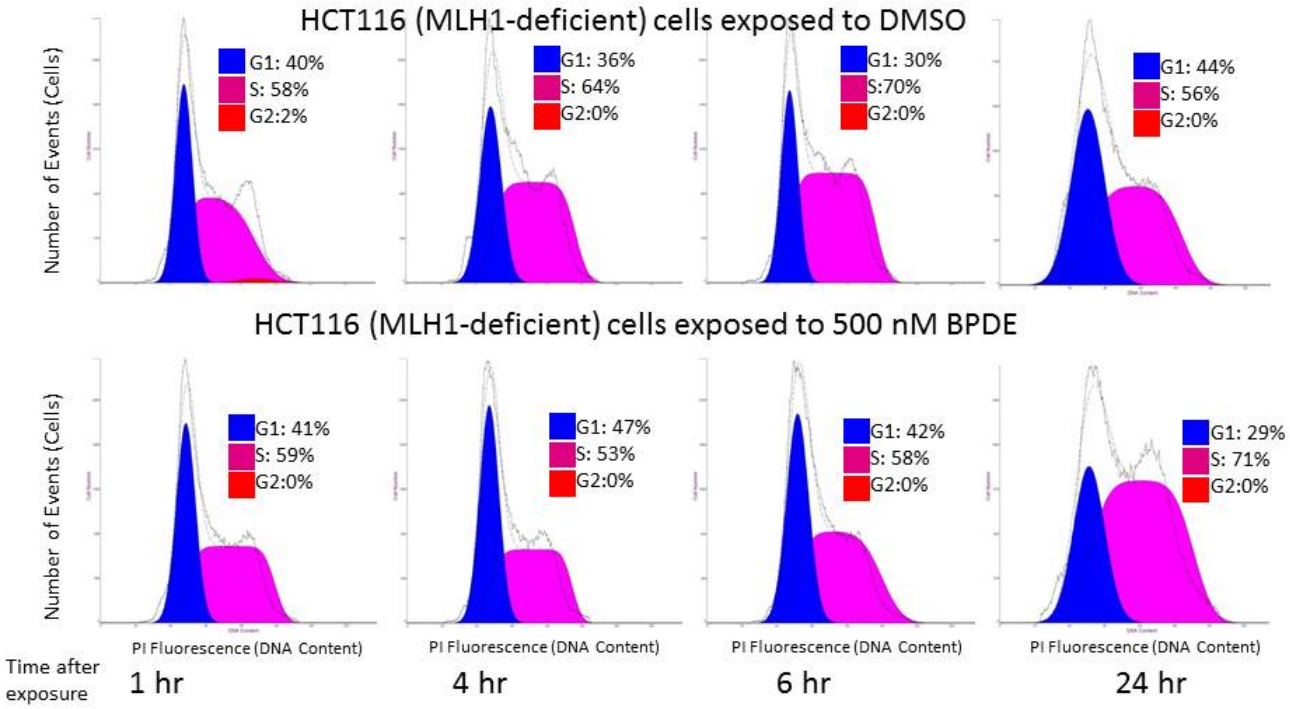

Figure 7b. Cell-cycle analysis of DMSO- and BPDE-exposed HCT116 cells. DNA content of HCT116 (MLH1-deficient) cells was measured via flow cytometry. HCT116 (MLH1-deficient) cells were exposed to 500 nM BPDE or DMSO for 1 hour. Cells were harvested at the indicated time periods following exposure. Labels indicate the percentage of the cell populations in each of the phases G1, S and G2.

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