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Mouse embryonic stem cells carrying one or two defective *Msh2* alleles respond abnormally to oxidative stress inflicted by low-level radiation

(DNA mismatch repair/oxidative DNA damage/ionizing radiation/apoptosis/mutagenesis)

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ABSTRACT **Chronic oxidative stress may play a critical role in the pathogenesis of many human cancers. Here, we report that mouse embryonic stem (ES) cells deficient in DNA mismatch repair responded abnormally when exposed to low levels of ionizing radiation, a stress known to generate oxidative DNA damage. ES cells derived from mice carrying either one or two disrupted *Msh2* alleles displayed an increased survival following protracted exposures to low-level ionizing radiation as compared with wild-type ES cells. The increases in survival exhibited by ES cells deficient in DNA mismatch repair appeared to have resulted from a failure to efficiently execute cell death (apoptosis) in response to radiation exposure. For each of the ES cell types, prolonged low-level radiation treatment generated oxidative genome damage that manifested as an accumulation of oxidized bases in genomic DNA. However, ES cells from *Msh2*$^{+/-}$ and *Msh2*$^{-/-}$ mice accumulated more oxidized bases as a consequence of low-level radiation exposure than ES cells from *Msh2*$^{+/+}$ mice. The propensity for normal cells with mismatch repair enzyme deficiencies, including cells heterozygous for inactivating mismatch repair enzyme gene mutations, to survive promutagenic genome insults accompanying oxidative stresses may contribute to the increased cancer risk characteristic of the hereditary nonpolyposis colorectal cancer syndrome.**

Inheritance of mutant alleles for genes encoding human DNA mismatch repair enzymes appears to confer a high lifetime risk of cancer development. For families with the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome, which is attributable to defects in one of four human DNA mismatch repair genes (*hMSH*2, *hMLH*1, *hPMS*1, and *hPMS*2), increased frequencies of carcinomas of the colon, endometrium, ovary, stomach, small intestine, pancreas, biliary tract, ureter, and renal pelvis have been reported (1–5). Carcinomas of the colon are particularly common among HNPCC kindreds; as many as 90% of carriers of mutant mismatch repair enzyme gene alleles may ultimately suffer with colorectal cancer (2). Abnormal DNA mismatch repair, which manifests as widespread somatic changes in the lengths of DNA microsatellite repeat sequences distributed throughout the genome, constitutes a characteristic feature of HNPCC syndrome tumor cell DNA (6–9). In cancer cells displaying microsatellite repeat length instability, the inherited defect in a DNA mismatch repair enzyme gene is usually accompanied by a somatic lesion in the remaining mismatch repair enzyme gene allele (3–5, 10, 11). However, although inactivation of both mismatch repair gene alleles may be required at some stage for the development of HNPCC tumors, whether normal cells heterozygous for mutant DNA mismatch repair enzyme genes exhibit mismatch repair deficiencies that might contribute to the initial stage(s) of HNPCC tumorigenesis has not been resolved (12).

Environmental factors, particularly dietary components, have been implicated as major determinants of sporadic colorectal cancer development (13, 14). The impact of such factors on colorectal tumorigenesis in HNPCC kindreds has not been ascertained. Nonetheless, a clue as to how environmental agents might promote mutations among cells with DNA mismatch repair defects has been provided by studies of DNA damage tolerance associated with mismatch repair enzyme deficiency. Treating a variety of DNA mismatch repair-deficient mammalian cells with alkylating agents such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine results in substantially less cell killing than that observed for mismatch repair competent cells (15–18). There is conflicting data regarding the response of cells with defective DNA mismatch repair to both UV and high dose-rate ionizing radiation (19–21). Nevertheless, the possibility that mismatch repair-deficient cells might exhibit improved survival on exposure to promutagenic stresses has profound mechanistic implications for tumorigenesis. Clearly, cell death constitutes a major barrier to neoplastic transformation caused by mutagens by the elimination of these DNA-damaged cells from continued growth. As such, cells with DNA mismatch repair enzyme deficiencies that fail to die as a consequence of mutagen exposure may be exquisitely vulnerable to neoplastic transformation by endogenous or exogenous promutagenic DNA-damaging agents.

A major promutagenic threat faced by colonic epithelial cells is that of chronic oxidative stress. Although reactive oxygen species arise in most cells as a result of normal metabolic processes, additional sources of oxidative stress in colonic cells may include enzymes such as cyclooxygenase 2 (COX-2) (22). High levels of COX-2 expression have been detected in human colorectal polyps, which are lesions that can progress to colorectal carcinomas (23), and a substantial body of evidence has accumulated that suggests that COX-2 might play a critical role in colorectal tumor development. First,

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Abbreviations: ES, embryonic stem; wt, wild type; HNPCC, hereditary nonpolyposis colorectal cancer; COX-2, cyclooxygenase 2; SIM, selected-ion monitoring; ECD, electrochemical detection; dGua, deoxyguanosine; 8OHdGua, 8-hydroxydeoxyguanosine; 6-TG, 6-thioguanine.

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treatment of people with familial adenomatous polyposis attributable to inherited *APC* gene mutations with sulindac, a COX inhibitor, has resulted in a reduction in polyp number and polyp size (24). In a similar fashion, treatment of mice with polyposis attributable to disrupted *Apc* genes with a specific COX-2 inhibitor also has attenuated colorectal polyp formation (25). Finally, mice with disrupted *Apc* genes have been found to develop fewer polyps if they also carry disrupted *Ptgs2* genes (encoding mouse COX-2) (25). Reactive oxygen species inflict damage on many cellular components, including genomic DNA (26). Genome lesions accompanying oxidative agent exposure include oxidized bases, broken DNA strands, and DNA–protein crosslinks (26, 27). Oxidative agent treatment of most cells also causes cell death. Because cells deficient in DNA mismatch repair appear tolerant to the lethal effects of alkylating agent exposure (15–18), we suspected that such cells might also be tolerant to the lethal effects of oxidating agent exposure. We reasoned that if mismatch repair-deficient cells failed to die when exposed to a prolonged oxidative stress, such cells might be prone to mutations that might promote neoplastic transformation. By this mechanism, chronic oxidative stress might play a critical role in HNPCC tumorigenesis.

MATERIALS AND METHODS

Embryonic Stem (ES) Cell Lines. The preparation, isolation, and characterization of $Msh2^{+/+}$, $Msh2^{+/-}$, and $Msh2^{-/-}$ ES cells has been described (18). ES cells were propagated in an undifferentiated state on mitotically inactive mouse embryo fibroblasts by using a growth medium (GMEM; Life Technologies, Grand Island, NY) supplemented with L-glutamine, sodium pyruvate, nonessential amino acids, 2-mercaptoethanol, murine leukemia inhibitory factor (Life Technologies), and 10% fetal bovine serum (ES cell-qualified; Life Technologies).

Ionizing Radiation Exposure and Clonogenic Survival Assessment. Subconfluent cultures of ES cells growing in the absence of feeder layer fibroblasts were sealed in gelatin-coated cell culture flasks and exposed to low-level ionizing radiation (0.004 Gy/min) by using a ^{137}Cs low dose-rate irradiator or exposed to acute dose radiation (1 Gy/min) by using a gamma cell 40 ^{137}Cs irradiator (Atomic Energy of Canada, Ottawa). For low-level radiation exposure, sealed flasks were maintained at 37°C in the low-dose irradiator for ≤72 h. As controls, unirradiated flasks were also sealed and incubated at 37°C for ≤72 h. Following irradiation, cultures were incubated under normal growth conditions for 14 days. To assess clonogenic survival, cultures were fixed in 50% methanol and stained with crystal violet as described (28). Colonies containing at least 50 cells were counted.

Mutation Analysis. To ascertain differences in the prevalence of *Hprt* mutant subclones among populations of ES cells with and without disrupted *Msh2* genes that were attributable to low-level radiation exposure, subconfluent cell cultures were irradiated in sealed culture flasks by using the low-dose irradiator for 72 h. Unirradiated flasks that were similarly sealed and incubated for 72 h served as controls. After radiation exposure, the ES cell cultures were incubated for 3–4 days (>2 population doublings) before being replated and subjected to selection in 5 μg/ml of 6-thioguanine (6-TG) for 10 days. Replated cultures were also incubated in complete media in the absence of 6-TG for 10 days to monitor plating efficiency. Surviving colonies were fixed, stained, and counted as described above. The prevalence of *Hprt* mutant subclones in control and irradiated cells was estimated as the number of 6-TG-resistant colonies per number of cells plated (corrected for plating efficiency).

Terminal Transferase Assay for Assessment of Apoptosis. To assess induction of apoptosis by protracted exposure to low-level radiation, sealed subconfluent cultures of the various ES cell lines were irradiated for 24 h as described above. Unirradiated sealed cultures incubated for 24 h served as controls. The appearance of apoptotic cells was monitored by using a terminal deoxynucleotidyltransferase assay as described (29, 30). Briefly, irradiated cells were fixed in 1% formaldehyde, permeabilized by suspension in 70% methanol, and then treated with terminal transferase in the presence of biotinylated deoxyUTP to end-label apoptotic DNA fragments. For analysis, the cells were stained with fluorescein isothiocyanate-avidin and propidium iodide and then subjected to flow cytometry (EPCS 752, Coulter Electronics). The fractions of apoptotic cells (cells end-labeled with terminal transferase) present in irradiated and in unirradiated ES cell cultures were estimated by using MDAD II data analysis software (Coulter).

GC/MS with Selected-Ion Monitoring (SIM). Genomic DNA was isolated and purified from ES cell line cultures subjected to low-level radiation exposure for 72 h and from control unirradiated ES cell cultures by using an ASAP genomic DNA isolation kit (Boehringer Mannheim). The recovered DNA was precipitated by using isopropanol and then stored at −70°C in 70% ethanol. GC/MS-SIM analyses for the presence of oxidized guanine and adenine bases as well as for thymine glycol and 5-methylcytosine in the DNA samples were performed as described (31). Samples were first hydrolyzed in 60% formic acid to obtain intact and modified bases and then treated with a solution of 99% bis(trimethylsilyl)trifluoroacetamide and 1% trichloromethylsilane dissolved in acetonitrile to convert the bases into volatile derivatives. To monitor the efficiency of base derivatization, samples were spiked with known quantities of the modified bases 8-azaguanine, 8-azaadenine, and 6-azathymine before acid hydrolysis. The base derivatives were analyzed by GC by using a Hewlett–Packard 5890 gas chromatograph with a Hewlett–Packard 5970 mass selective detector.

HPLC with Electrochemical Detection (ECD). Isolation and purification of DNA from control and irradiated ES cells was performed as described above. Genomic DNA samples were then treated with nuclease P1 and alkaline phosphatase as described (32, 33) and analyzed for the presence of 8-hydroxydeoxyguanosine (8OHdGua) by HPLC-ECD (33). 8OHdGua and deoxyguanosine (dGua) were separated on a Waters 510 HPLC and quantified by using a Bioanalytical Systems LC-4C electrochemical detector (West Lafayette, IN) and a Beckman 160 UV detector, respectively. Levels of 8OHdGua/10^6 dGua were calculated by converting the detected 8OHdGua and dGua peak areas to amounts based on standard curves of the authentic standards 8OHdGua (Cayman Chemicals, Ann Arbor, NH) and dGua (Sigma). For 8OHdGua standards, HPLC-ECD peak areas were a linear function of 8OHdGua amounts across a range of 200 to >800 fmol (r^2 = 0.995). All genomic DNA samples contained 8OHdGua amounts (>250 fmol), permitting measurement in the linear range of the HPLC-ECD assay.

RESULTS AND DISCUSSION

To determine whether cells containing DNA mismatch repair enzyme gene defects were tolerant to prolonged oxidative stress, we treated ES cells from mice carrying disrupted *Msh2* genes (18) with protracted exposures to low levels of ionizing radiation and then assessed clonogenic survival. A previous analysis of alkylation agent effects on mismatch repair-deficient ES cells revealed that ES cells carrying two disrupted *Msh2* alleles demonstrated improved survival on treatment with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine as compared with ES cells carrying one or two normal *Msh2* alleles (18). In a similar fashion, $Msh2^{-/-}$ ES cells exhibited markedly improved survival following low-level radiation exposure when

compared with $Msh2^{+/+}$ cells (Fig. 1*A*). These data indicated that cells devoid of wild-type (wt) *Msh2* genes were tolerant to the lethal effects of both alkylating agents and oxidating agents. Surprisingly, $Msh2^{+/-}$ ES cells also exhibited improved low-level radiation survival (Fig. 1*A*). Thus, although ES cells heterozygous for defective *Msh2* alleles were not tolerant to the lethal effects of alkylating agents, such cells appeared tolerant to the lethal effects of oxidating agents. Of note, although ES cells containing disrupted *Msh2* genes may be tolerant to the lethal effects of prolonged low-level radiation exposure, such cells may not be intrinsically resistant to killing by acute high-level ionizing radiation treatment. When each of the ES cell lines was treated with brief exposures to ionizing radiation at a high dose rate similar to that used for cancer treatment, only slight differences in clonogenic survival attributable to defective *Msh2* genes were detected (Fig. 1*B*).

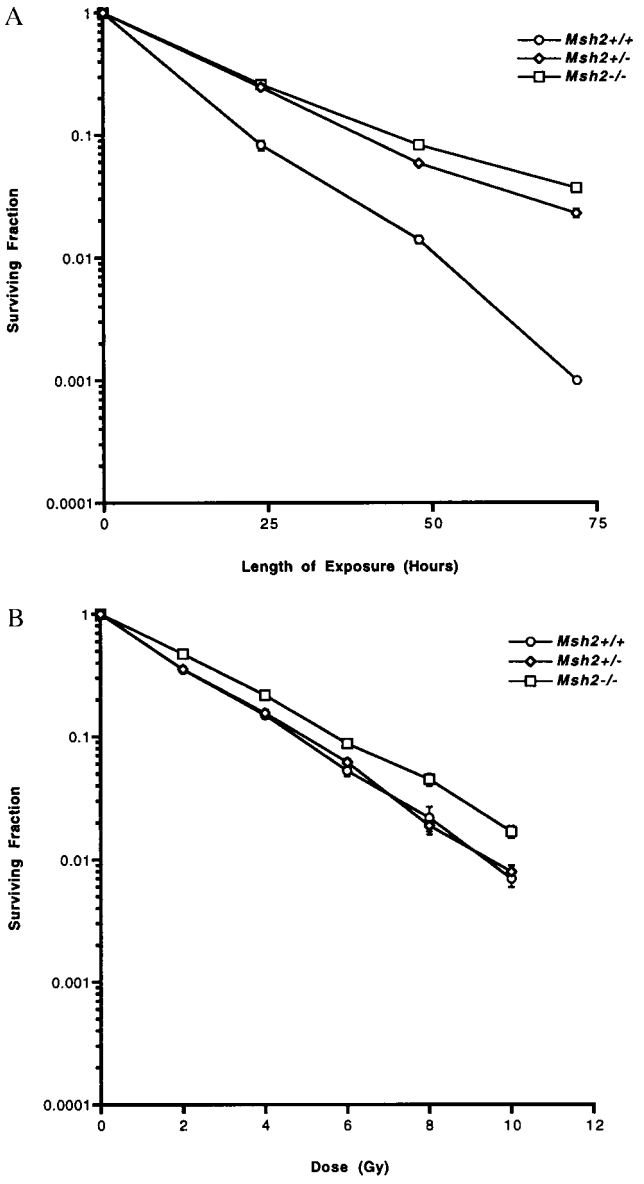

FIG. 1. Mouse ES cells carrying either one or two disrupted *Msh2* genes appear tolerant to lethal effects of low-level radiation exposure. Displayed are clonogenic survival curves obtained from analysis of wt mouse ES cells ($Msh2^{+/+}$) and ES cells carrying disrupted *Msh2* genes ($Msh2^{+/-}$ and $Msh2^{-/-}$) after (*A*) prolonged (24, 48, and 72 h) exposure to low-level ionizing radiation (0.004 Gy/min) or after (*B*) treatment with acute dose ionizing radiation (1.0 Gy/min). Each symbol represents the mean of multiple, triplicate experiments; the SEM is shown by using error bars. In some cases, the error bars are smaller than the symbol.

Mammalian cells suffering extensive genome damage frequently activate signal transduction pathways culminating in apoptosis (34). The appearance of internucleosomal DNA fragments in dying cells is one of the hallmark features of cellular apoptosis. When we exposed each of the different ES cell lines to low-level ionizing radiation, internucleosomal DNA fragments were evident among isolated genomic DNA as assessed by using agarose gel electrophoresis (data not shown). To ascertain whether differences in clonogenic survival between wt ES cells and ES cells carrying disrupted *Msh2* alleles following prolonged exposure to low levels of ionizing radiation might be attributable to differences in the propensity for such cells to undergo apoptosis, cells from each of the ES lines were treated with low-level radiation and then assayed for the genome fragmentation characteristic of apoptosis (29, 30). By using flow cytometry to monitor the appearance of cells containing fragmented DNA that can be end-labeled by using terminal transferase, substantial increases in the prevalence of apoptotic cells were evident among $Msh2^{+/+}$ ES cells exposed to low levels of radiation for 24 h (Fig. 2). In contrast, minimal increases in apoptotic cells were detected in populations of ES cells containing disrupted *Msh2* alleles following irradiation (Fig. 2). The failure of ES cells with one or two defective *Msh2* genes to efficiently execute apoptosis may be one mechanism by which these *Msh2*-deficient cells enjoyed increased clonogenic survival compared with wt ES cells following protracted low-level radiation exposure (Fig. 1*A*).

Like other oxidative stresses, exposures to ionizing radiation generate a variety of genome injuries in treated cells (26, 27). Mammalian cells exposed to ionizing radiation also frequently display increased mutations (35, 36). Precisely which genome lesion(s) lead to mutations has not been firmly established (31). Nonetheless, oxidation of bases in genomic DNA has been detected following ionizing radiation treatment, and the oxidized base 8-hydroxyguanine has been found to be promutagenic, perhaps by leading to mispairing during DNA replication (37–39). When wt ES cells and ES cells carrying disrupted *Msh2* alleles were assessed for the presence of oxidized bases in DNA isolated following low-level radiation exposure, increases in 8OHdGua were detected in cellular

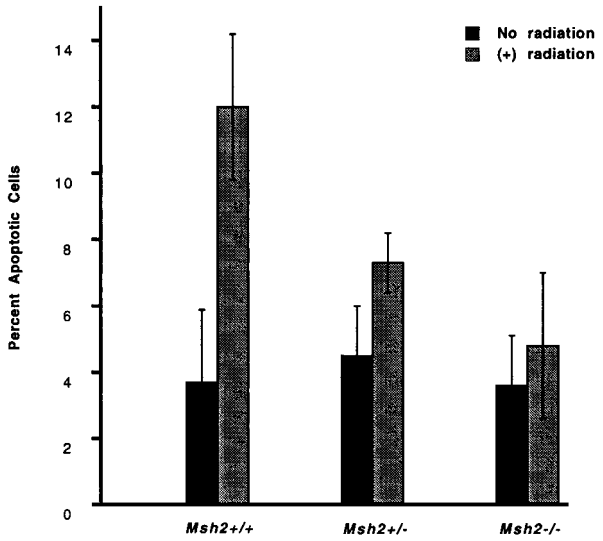

FIG. 2. Mouse ES cells containing defective *Msh2* genes fail to efficiently execute cell death (apoptosis) in response to low-level radiation treatment. Apoptosis induction was assessed by using flow cytometry to monitor the appearance of cells containing fragmented DNA (see *Materials and Methods*) for wt ES cells ($Msh2^{+/+}$) and for ES cells carrying disrupted *Msh2* genes ($Msh2^{+/-}$ and $Msh2^{-/-}$) after exposure to low-level ionizing radiation for 24 h. Mean values ± SEM are displayed.

DNA from each of the ES lines by using HPLC-ECD (Fig. 3*A*). Furthermore, greater increases in 8OHdGua levels attributable to low-level radiation exposure were detected in ES cells containing defective *Msh2* genes as compared with wt ES cells. Higher levels of 8-hydroxyguanine in irradiated ES cells carrying disrupted *Msh2* alleles were confirmed by GC/MS-SIM analysis (data not shown). In addition, elevated levels of 8-hydroxyadenine and thymine glycol were detected following low-level irradiation of $Msh2^{-/-}$ cells compared with $Msh2^{+/+}$ cells when analyzed by GC/MS-SIM (Fig. 3*B*). As expected, not all bases were affected by irradiation, as evidenced by the lack of change in 5-methylcytosine levels (Fig. 3*B*).

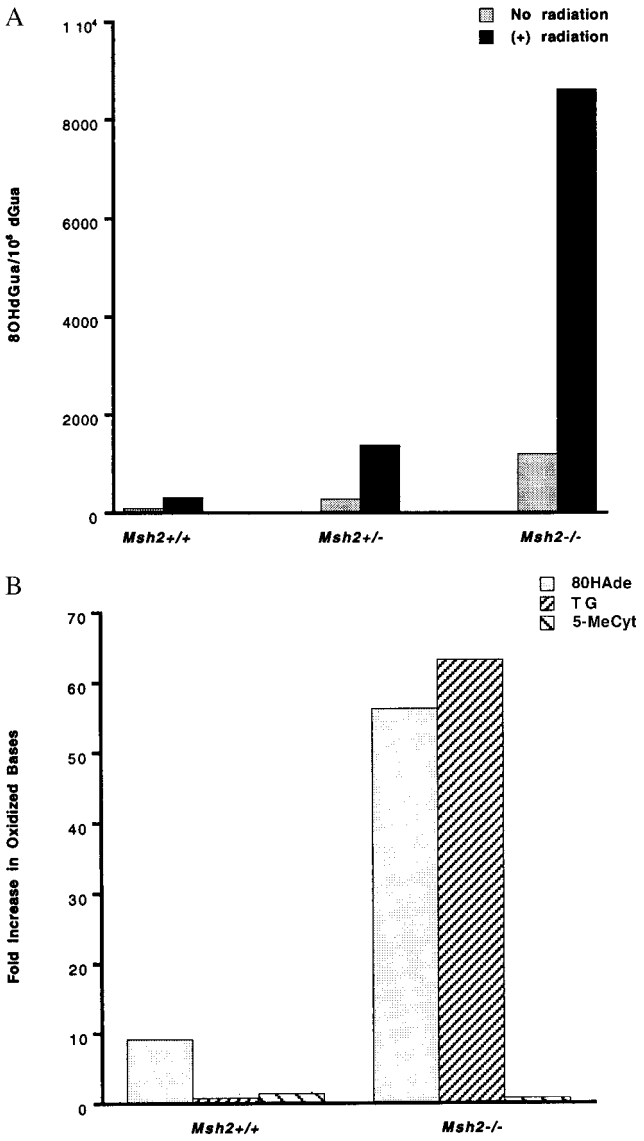

FIG. 3. DNA from mouse ES cells containing disrupted *Msh2* genes accumulates more oxidative base damage as a consequence of low-level radiation exposure than wt ES cells. (*A*) Increases in oxidized dGua (8OHdGua) in DNA from different ES cell lines treated with low-level radiation, assessed by using HPLC-ECD (see *Materials and Methods*), are displayed as a ratio of the number of detected 8OHdGua per 10^6 dGua in irradiated vs. unirradiated cell cultures. (*B*) Levels of the oxidized bases 8-hydroxyadenine (8OHAde) and thymine glycol (TG) as well as levels of 5-methylcytosine (5-MeCyt) in DNA from ES cells with and without defective mismatch repair (i.e., $Msh2^{+/+}$ and $Msh2^{-/-}$, respectively) were determined before and after low-level irradiation by GC/MS-SIM (see *Materials and Methods*). For both HPLC-ECD and GC/MS-SIM, DNA from ES cells was collected and pooled following at least five independent exposures before being subjected to analysis for oxidized bases.

Recent studies of ethylnitrosourea-induced lymphomagenesis in $Msh2^{-/-}$ vs. $Msh2^{+/+}$ mice have suggested that mutagen-associated tumorigenesis may be significantly modulated by mismatch repair enzyme function (40). To determine whether the oxidative stress generated by low-level radiation exposure caused mutations as well as oxidative DNA damage, the prevalence of 6-TG-resistant *Hprt* mutants among ES cells from each of the lines was determined before and after protracted low-level radiation treatment. In the absence of low-level radiation treatment, 6-TG-resistant clones were not detected among populations of $Msh2^{+/+}$ or $Msh2^{+/-}$ ES cells; however, fairly high numbers of 6-TG-resistant clones were detected in populations of $Msh2^{-/-}$ ES cells (Table 1). The high prevalence of *Hprt* mutant clones among mouse ES cells homozygous for disrupted *Msh2* alleles was consistent with previous reports demonstrating spontaneous *HPRT* mutation rates as high as 1.5 in 10^5 per generation in human cancer cells with mismatch repair gene deficiencies (41, 42). When the prevalence of 6-TG-resistant clones was assessed for each of the ES cell lines following protracted low-level radiation treatment, even higher numbers of mutant clones were detected among populations of $Msh2^{-/-}$ ES cells. Mutant clones were also detected among populations of irradiated $Msh2^{+/-}$ ES cells (Table 1). Whether this represents an increase in mutation frequency in $Msh2^{+/-}$ ES cells attributable to radiation exposure is not entirely clear however, as the number of *Hprt* mutant clones detected was low and at the limit of detection of the 6-TG resistance mutation assay used. Finally, when we exposed populations of $Msh2^{+/+}$ ES cells to low-level ionizing radiation, no 6-TG-resistant clones were detected (Table 1).

All of the data we have collected here demonstrate that mouse ES cells containing defective DNA mismatch repair enzyme genes respond abnormally to chronic oxidative stress inflicted by protracted low-level radiation exposure. When compared with $Msh2^{+/+}$ ES cells, $Msh2^{-/-}$ ES cells exhibited increased clonogenic survival, decreased apoptosis, increased oxidative genome damage, and increased mutations following low-level radiation treatment. These results implicate DNA mismatch repair enzymes as critical participants in cellular responses to oxidative stresses. The increases in *Hprt* mutations detected among $Msh2^{-/-}$ ES cells following prolonged exposure to low-level radiation suggest that, in addition to participation in the repair of base mismatches and in transcription-coupled repair (21, 43), DNA mismatch repair enzymes may function in some way to facilitate the repair of bases suffering oxidation damage. To do so, the enzymes may act to recognize and repair mismatches arising from mispairing of oxidized bases on template DNA strands during DNA replication (43). This action may be similar to the role played by the DNA mismatch repair system in the recognition and binding of alkylating agent- and cisplatin-induced DNA adducts that, in part, modulates cytotoxicity (44). The propensity for ES cells carrying disrupted *Msh2* genes to accumulate greater amounts of oxidized guanine and adenine bases in genomic DNA than wt ES cells as a consequence of low-level radiation exposure also supports the possibility that DNA mismatch repair en-

Table 1. Frequency of 6-TG resistance

Cell line	No radiation	radiation
$Msh2^{+/+}$	ND ($<2.7 \times 10^{-6}$)	ND ($<3.6 \times 10^{-7}$)
$Msh2^{+/-}$	ND ($<1.7 \times 10^{-6}$)	1.5×10^{-6} ($\pm 0.7 \times 10^{-6}$)
$Msh2^{-/-}$	5×10^{-4} ($\pm 0.7 \times 10^{-4}$)	2×10^{-3} ($\pm 0.4 \times 10^{-3}$)

Prevalence of 6-TG-resistant clones in ES cell cultures before and after protracted low level radiation exposure was assessed as described in *Materials and Methods*. ND, no 6-TG-resistant colonies detected.

zymes may play some sort of role in oxidative DNA damage repair. However, neither the increases in *Hprt* mutations nor the increases in oxidized bases in the mismatch repair-deficient cells necessarily implicate DNA mismatch repair enzymes as direct participants in the recognition and repair of oxidized DNA *per se*. The data are also consistent with indirect contributions to cellular oxidative stress responses.

The oxidation damage tolerance displayed by $Msh2^{-/-}$ cells relative to $Msh2^{+/+}$ ES cells, which is evidenced by decreased apoptosis and increased clonogenic survival despite increased accumulation of oxidative DNA damage, raises the possibility that DNA mismatch repair enzymes might contribute to cell fate decision pathways, such as those leading to cell death, that are normally executed as a consequence of oxidative stress. The alkylation damage tolerance characteristic of DNA mismatch repair-deficient cells has been proposed to result from the absence of a futile alkylation damage-repair process involving DNA mismatch repair enzymes (45). In mismatch repair-competent cells, but not mismatch repair-deficient cells, this futile repair process may lead to DNA strand breaks and cell death. Perhaps oxidation damage tolerance may result from a similar absence of futile repair processes. Another explanation for diminished vulnerability to cell death accompanying DNA repair enzyme deficiency may be that DNA repair itself directly or indirectly generates signals that drive cell fate decision pathways, including apoptosis. For example, in many normal mammalian cells, the cell fate regulator p53 transduces signals leading to G_1 cell cycle arrest or apoptosis in response to DNA damage (28). However, following UV light treatment, the appearance of photodamaged bases does not appear sufficient to trigger p53 increases; rather, DNA strand breaks that arise during DNA repair are required for p53 induction, underscoring a close association between DNA repair and p53-dependent cell fate signaling (46). In support of this concept, functional interactions between p53 and the repair helicases XPB and XPD have been reported (47). Perhaps attempted repair of oxidized base damage by a process involving DNA mismatch repair enzymes also initiates a signal transduction pathway culminating in cell death. Failure to attempt repair may result in a failure to rapidly trigger apoptosis, permitting long-term adaptive responses to oxidative damage to emerge (48).

Perhaps the most striking data we have presented here concern the oxidation tolerance displayed by ES cells heterozygous for disrupted *Msh2* alleles. Clearly, if epithelial cells in an organ such as the colon in HNPCC carriers heterozygous for mutant DNA mismatch repair genes also display increased survival in response to promutagenic oxidative stresses, such cells will be at high risk for neoplastic transformation. Some mismatch repair deficiencies in human cells heterozygous for mutant DNA mismatch repair genes have been attributed to possible "dominant-negative" actions of defective mismatch repair enzymes encoded by the mutant alleles (12). Mismatch repair deficiency in cells heterozygous for mutant DNA mismatch repair genes might also result from acquired damage to normal mismatch repair gene alleles. These explanations appear unlikely to account for the improved survival exhibited by $Msh2^{+/-}$ ES cells exposed to low-level radiation, because $Msh2^{+/-}$ ES cells appeared not only oxidation damage-tolerant, like $Msh2^{-/-}$ ES cells, but also alkylation damage-sensitive, like $Msh2^{+/+}$ ES cells (18). Thus, normal cells heterozygous for mutant DNA mismatch repair genes may be vulnerable to some promutagenic stresses but not others. Nevertheless, most cancers from HNPCC carriers contain both an inherited mutation of one DNA mismatch repair gene allele and an acquired genome lesion affecting the remaining allele, suggesting that a selective advantage for homozygous inactivation of DNA mismatch repair enzyme genes may arise during carcinogenesis (3–5, 10, 11). In our studies, perhaps some of the $Msh2^{+/-}$ ES cells may have suffered damage or loss of normal mismatch repair enzyme alleles as a consequence of prolonged low-level radiation exposure. Although this mechanism does not account for the propensity for $Msh2^{+/-}$ ES cells to survive prolonged oxidative stress like that inflicted by low-level radiation, the possible appearance of a small number of mismatch repair-deficient cells among irradiated $Msh2^{+/-}$ ES cells may explain the detection of *Hprt* mutant clones among irradiated $Msh2^{+/-}$ ES cells in our study. de Wind *et al.* (40) have presented data indicating that $Msh2^{+/-}$ mice suffer increased numbers of spontaneous tumors when compared with $Msh2^{+/+}$ littermates. However, in their studies, spontaneously arising tumors in $Msh2^{+/-}$ mice rarely displayed either loss of the second *Msh2* allele or microsatellite instability (40). We speculate that normal cells heterozygous for mutant DNA mismatch repair gene alleles may be at increased risk for neoplastic transformation by virtue of an increased tendency to survive exposure to promutagenic chronic oxidative stress. The progeny of these heterozygous cells, which may or may not have also acquired a somatic lesion in the remaining normal DNA mismatch repair gene allele, may be at increased risk for malignant progression both by virtue of an increased tendency to survive exposure to promutagenic environmental stresses causing either DNA oxidation or DNA alkylation and by virtue of an increased tendency for spontaneous mutation and DNA microsatellite repeat length changes (49, 50). If this model is true, amelioration of oxidative stress, perhaps via inhibition of oxidative enzymes such as COX-2, may attenuate the earliest steps in colorectal carcinogenesis for HNPCC carriers.

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