Msh2 status modulates both apoptosis and mutation frequency in the murine small intestine

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ABSTRACT   Deficiency in genes involved in DNA mismatch repair increases susceptibility to cancer, particularly of the colorectal epithelium. Using *Msh2* null mice, we demonstrate that this genetic defect renders normal intestinal epithelial cells susceptible to mutation *in vivo* at the *Dlb-1* locus. Compared with wild-type mice, *Msh2*-deficient animals had higher basal levels of mutation and were more sensitive to the mutagenic effects of temozolomide. Experiments using *Msh2*-deficient cells *in vitro* suggest that an element of this effect is attributable to increased clonogenicity. Indeed, we show that *Msh2* plays a role in the *in vivo* initiation of apoptosis after treatment with temozolomide, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, and cisplatin. This was not influenced by the *in vivo* depletion of O^6-alkylguanine-DNA-alkyltransferase after administration of O^6-benzylguanine . By analyzing mice mutant for both *Msh2* and *p53*, we found that the *Msh2*-dependent apoptotic response was primarily mediated through a *p53*-dependent pathway. *Msh2* also was required to signal delayed *p53*-independent death. Taken together, these studies characterize an *in vivo Msh2*-dependent apoptotic response to methylating agents and raise the possibility that *Msh2* deficiency may predispose to malignancy not only through failed repair of mismatch DNA lesions but also through the failure to engage apoptosis.

Structural distortions produced by nucleotides that are either unpaired or paired with noncomplementary nucleotides are recognized by proteins encoded by the mismatch repair genes. Several members of this family of genes have been characterized within *Saccharomyces cerevisiae*, which led to the identification of mammalian homologues. Six human mismatch repair genes have been cloned: *MSH2*, *MLH1*, *PMS1*, *PMS2*, *MSH3*, and *GTBP* (1, 2). Constitutive inactivation of these genes has been associated with the development of cancer, the best characterized relationship being between the inherited cancer-susceptibility syndrome of hereditary nonpolyposis colorectal cancer and germline mutations in *MSH2*, *MLH1*, and *PMS2* (2, 3). Mice have been produced bearing targeted inactivations of the *Mlh1*, *Msh2*, *Pms1*, and *Pms2* genes (4, 5). Homozygous mice from all of these mutant strains are viable but prone to the development of different types of neoplasia, predominantly of the lymphoid and intestinal lineages.

MSH2 recognizes mismatched nucleotides as a heterodimer with *GTBP* (6). In what is assumed to be a direct consequence of failure of this process, microsatellite instability is increased in murine *Msh2* −/− cells (4). These data support the concept that failed recognition and repair of mismatch lesions leads directly to an increase in mutation frequency and thereby to malignancy. However, the consequences of *MSH2* deficiency are complex, with *Msh2* −/− cells also showing hyperrecombination and increased survival after exposure to methylating agents *in vitro* (4). This last property suggests that *MSH2* status may affect the ability to undergo apoptosis, with increased resistance to methylating agents arising from failure to detect damage and hence initiate apoptosis. Results from several different tumor cell lines deficient in mismatch repair are consistent with this hypothesis (7). We have used embryonic stem (ES) cells and mice bearing a gene-targeted inactivation of *Msh2* (4) to investigate the *in vivo* role of this protein in the prevention of mutation and in the initiation of apoptosis after DNA damage. We find that, in the murine small intestine, *Msh2* deficiency reduces the apoptotic response to temozolomide, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), and cisplatin. We also show that this response is normally *p53*-dependent. In the absence of *Msh2*, we find a dose-dependent increase in the *in vivo* mutation frequency, and we have performed *in vitro* experiments that suggest that a component of this increase may arise from the failure to engage death pathways.

METHODS

Mice. All of the mice used in these studies were derived from a single outbred colony segregating for Ola/129, BALB/c, and SWR genomes. The percentage contribution of each genome type was not determined for each experimental cohort, but, wherever possible, littermate controls were used to minimize any background-dependent effect. The apoptosis studies described here have been minimally reproduced on an inbred C57BL/6 background, and similar results have been obtained (data not shown). The *Dlb-1* assay was performed as described (8). The *Dlb-1* assay is a specific locus assay in which somatic mutations are detected that inactivate the polymorphic genetic locus, *Dlb-1*, which encodes a lectin binding site. The assay is autosomal with the locus located on mouse chromosome 11. Two alleles of *Dlb-1* have been characterized. The *Dlb-1b* allele specifies binding of the Dolichos biflorus agglutinin to intestinal epithelium. The *Dlb-1a* allele specifies binding to the vascular endothelium. In mice that are *Dlb-1b/Dlb-1a*, any inactivating mutation of the single *Dlb-1b* allele leads to loss of the ability to bind the lectin. After cell proliferation and clonal expansion, mutations that occurred in the stem cell population

of the intestine result in the formation of a clone that does not bind a peroxidase conjugate of Dolichos biflorus agglutinin. These unstained clones are easily identified and quantified in stained wholemounts of the small intestine. To perform the *Dlb-1* assay, experimental cohorts were derived by backcrossing *Msh2*-deficient animals derived from the breeding colony described above to two different C57BL/6 strains, one of which was homozygous for the *Dlb-1a* allele and one of which was homozygous for the *Dlb-1b* allele. Mice subsequently were intercrossed from these two lines to generate mice heterozygous at the *Dlb-1* locus and segregating for all possible *Msh2* genotypes.

Genotyping Mice by PCR. Tail biopsies were boiled in 500 μl of lysis buffer (0.1M Tris, pH 8.5/0.005M EDTA/0.2% SDS/0.2M NaCl) for 15 min. A sample (1 μl) was used as template in a 50-μl three primer PCR reaction containing 5 μl of PCR buffer, 2.5 μl of 1% detergent, 2.5 μl each of primer (10 pmol/μl), 1 μl of dNTP (40 mM), 2 μl of MgCl$_2$ (50 mM), 30 μl of double-distilled water, and 1 μl of *Taq* (1.25 units) (GIBCO/BRL) (primer 1, CGGCCTTGAGCTAAGTCTAT-TATAAGG; primer 2, GGTGGGATTAGATAATGCCT-GCTCT; primer 3, CCAAGATGACTGGTCGTACATAA-G). Reaction conditions were 94°C for 5 min, 60°C for 2 min, and 72°C for 2 min (×1); 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min (×30); and 94°C for 1 min, 60°C for 2 min, and 72°C for 10 min (×1). Ten microliters of product was analyzed on a 4% Tris·Borate EDTA agarose gel with the wild-type allele generating a 164-bp product and the targeted allele generating a 194-bp product.

Reagents and Administration. Mice (8–12 weeks old) were given i.p. injections of MNNG (50 mg/kg body weight), temozolomide (100 mg/kg), cisplatin (10 mg/kg), or O^6-benzylguanine (BeG) (60 mg/kg). The volume for all reagents was 0.25 ml. All drugs, except cisplatin (David Bull Laboratories, Warwick, U.K.), were prepared fresh, were first dissolved in DMSO (10% vol/vol), and were made to a final concentration with PBS (temozolomide and BeG) or corn oil (MNNG). Mice were exposed to γ-irradiation by using a ^{137}Cs source at 0.27 Gy·min^{-1} for 15 min, so that each animal received a dose of 4 Gy.

Quantitation of Apoptosis. At each indicated time point after injection, a minimum of three animals were killed, and the small intestine was removed, was flushed with water, and was fixed overnight in methacarn (4 parts methanol, 2 parts chloroform, 1 part acetic acid). Histological sections were made, and apoptosis was scored as described (9). A minimum of 50 half crypts were scored per animal.

Cell Culture Experiments. ES cells were maintained under standard conditions in lymphocyte inhibitory factor-supplemented media. After electroporation with the PNT (10) vector, which contains both neomycin and herpes simplex virus thymidine kinase gene cassettes, clones were selected in 200 μg/ml G418. Clones were expanded and then were exposed to temozolomide in serum-free medium for 4 hours. Cells were subsequently maintained out of selection for a period of 4 days to permit endogenous levels of herpes simplex virus thymidine kinase to decline. Cells then were plated at a range of cell densities. Clonogenicity and mutation frequency were respectively scored by counting viable colonies after 14 days of culture in either normal medium or after selection in 2 mM ganciclovir.

RESULTS

***In Vivo* Mutation Frequency Scored at the *Dlb-1* Locus.** Andrew *et al* (11) recently demonstrated striking increases in mutation frequency in *Msh2*-deficient mice after treatment with *N*-methyl-*N*-nitrosourea by using a transgenic *Lac*I reporter system. We wished to address this same question specifically within the stem cell population of the murine

intestine, both at endogenous levels of DNA damage and also after treatment with the cancer chemotherapeutic drug temozolomide. The *in vivo* mutation frequency was scored at the *Dlb-1* locus within the crypt stem cells of the intestinal epithelium of animals that were wild-type, heterozygous, or null for *Msh2* and heterozygous (a/b) at the *Dlb-1* locus. Twenty days after exposure to Temozolomide, *Dlb-1* mutations were scored as non-lectin binding clones by using standard techniques (9, 12). Mutation frequencies in untreated and drug treated animals are shown in Fig. 1. Compared with wild-type mice, *Msh2* −/− mice were found to have a significantly increased frequency of mutations both at endogenous levels of damage and after temozolomide treatment ($P < 0.05$, Mann–Whitney U test).

Mutation Frequency and Clonogenicity *in Vitro*. The observed increase in mutation frequency may arise directly from failed DNA repair or as a consequence of increased survival of cells bearing DNA damage. It is currently not possible to determine the clonogenic survival of individual crypt stem cells because each crypt contains multiple stem cells and *in vivo* clonogenicity assays rely on ablation of this entire population. We have therefore addressed this question in an *in vitro* model system. We determined clonogenicity and mutation frequency in ES cells wild-type or mutant for *Msh2* that had been stably transfected with PNT (10), a vector encoding both the herpes simplex virus thymidine kinase gene and the neomycin phosphotransferase gene. The mutation frequency was scored at the thymidine kinase locus on the basis of resistance to the drug ganciclovir. Cultures were maintained under G418 selection to eliminate cells in which the entire vector had been inactivated: for example, after chromosome loss. After a 4-hour exposure to 2 mM temozolomide, 21% of *Msh2* −/− cells formed colonies. This figure was reduced to 1.57% in +/+ cells (these figures represent the mean clonogenicity from five different experiments, each performed in triplicate and corrected for the plating efficiency of untreated cells). The mutation frequency, as scored by ganciclovir resistance, was 1.002×10^{-3} in −/− cells and 9×10^{-6} in +/+ cells (mean values derived from three different experiments performed in
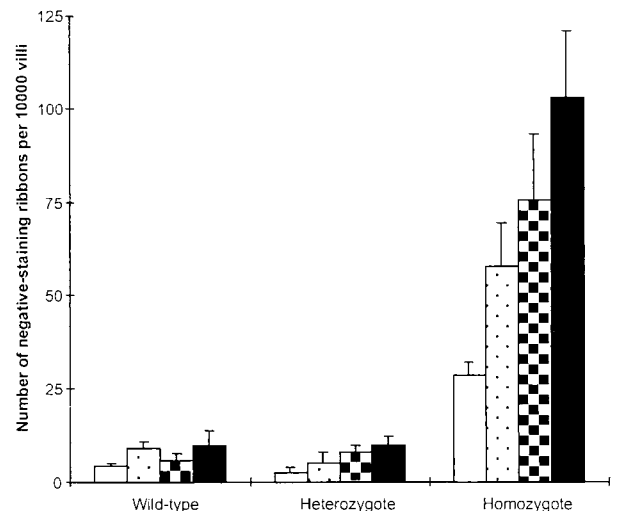

Fig. 1. *Dlb-1* mutation frequencies after exposure to temozolomide. Mutation frequencies were determined from intestinal wholemounts prepared 20 days after i.p. injection with a range of doses of temozolomide. Columns represent mean mutation frequency at the *Dlb-1* locus at the drug dosage shown. Error bars represent SEM. A minimum of three animals was scored per group. Open bars, 0 mg/kg; stippled bars, 25 mg/kg; hatched bars, 50 mg/kg; filled bars, 100 mg/kg. Under all conditions, *Msh2* −/− mice had significantly higher mutation frequencies compared with wild-type and heterozygote animals ($P < 0.05$, Mann–Whitney U test).

triplicate). These results show that *Msh2* deficiency confers a 13-fold increase in clonogenicity and a 110-fold increase in mutation frequency. A significant component of the *Msh2*-dependent elevation in mutation frequency is therefore directly attributable to increased survival of *Msh2* −/− cells.

***Msh2*-Dependent Apoptosis.** To test directly whether *Msh2* has a significant role in the induction of apoptosis *in vivo* after DNA damage, we analyzed the prevalence of apoptosis after exposure to the methylating agents MNNG and temozolomide. We similarly analyzed the effect of the cross-linking agent cisplatin caused by the recognized association between cisplatin resistance and mismatch repair deficiency and also because cisplatin 1,2 intrastrand adducts may be directly recognized by the *MSH2/GTBP* complex (13). Apoptosis was scored within the crypts of the small intestine. This system was chosen for three principal reasons: first, the known intestinal expression of *MSH2* (14); second, the demonstrated role of *Msh2* in prevention of intestinal neoplasia (15); and third, because apoptosis within the murine small intestine has been well characterized (9, 16) .

Apoptosis was scored histologically within the crypts of cohorts of mice that were either wild-type (+/+) or homozygous null (−/−) for *Msh2* over a 72-hour period after drug administration (Fig. 2). In *Msh2* +/+ animals, all of the drugs used induced measurable increases in intestinal apoptosis, and, in all cases, the peak induction of apoptosis was seen between 8 and 16 hours. In comparison to the pattern observed in the *Msh2* +/+ animals, the apoptotic response of *Msh2* −/− mice was significantly diminished for all three drug types ($P < 0.05$, Mann–Whitney U test). The *Msh2*-dependent decrease in intestinal apoptosis was greatest for temozolomide and least for cisplatin. In both *Msh2* +/+ and −/− mice, apoptosis occurred at peak levels within the replicative compartment of the crypt with the mode lying between cell positions 4 and 6, overlapping the stem cell region; thus, *Msh2* status influenced the number but not the spatial distribution of apoptosis within the crypt (data not shown).

Apoptosis Is Unaffected After BeG Administration. The DNA repair protein O^6-alkylguanine-DNA-alkyltransferase (AGT) functions by recognizing and removing specific alkyl lesions from DNA. Failure of AGT to repair O^6-methyl-G results in O^6-methyl-G:T mispairs after replication, and it is postulated that these mispairs mediate cell death via mismatch repair (7). AGT activity may therefore complicate these results by effectively removing methylation damage and so suppressing cell death such that the extent of *Msh2*-dependent apoptosis is underestimated. To investigate this possibility, we used the inhibitor BeG to deplete AGT before challenge with MNNG and temozolomide (17). Effective depletion of AGT activity was confirmed with no detectable AGT activity between 1 and 12 hours after BeG administration (Fig. 3*A*). No induction of apoptosis was associated with BeG treatment alone (data not shown). Apoptosis was scored in *Msh2* +/+ and −/− animals 6 hours after administration of MNNG and temozolomide and 7 hours after administration of BeG. At this time, apoptosis again was observed to depend on *Msh2* status, with a significant reduction observed in *Msh2* −/− mice after MNNG and temozolomide ($P < 0.05$, Mann–Whitney U test in both cases). The degree of *Msh2* dependency was not significantly different in non-BeG treated mice (Fig. 3*B*).

P53 Dependency. To determine whether the observed apoptosis was mediated by a *p53*-dependent pathway, we investigated intestinal apoptosis after drug administration in *p53* −/− mice. In contrast to *p53* +/+ mice, none of the drugs used induced apoptosis above background levels at 6 hours (Fig. 4*A*). Delayed *p53*-independent apoptosis was, however, observed from 24 to 96 hours after temozolomide administration in *p53* −/− mice (Fig. 4*B*). Mice doubly mutant for both *Msh2* and *p53* failed to show this delayed *p53*-independent apoptosis ($P < 0.001$, Mann–Whitney U test at 72
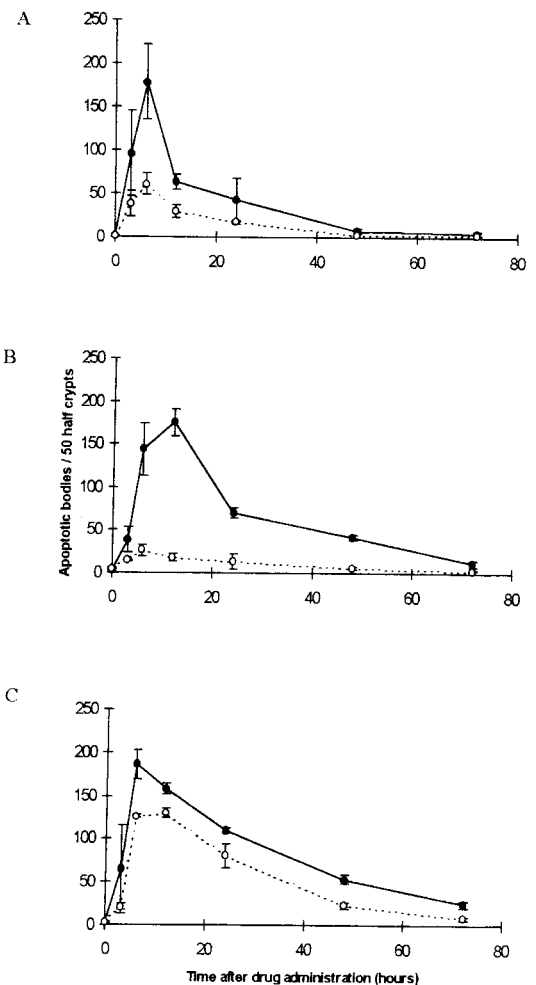

Fig. 2. The *in vivo* apoptotic response after drug exposure. Cohorts of *Msh2* +/+ or −/− animals were given i.p. injections of 50mg/kg MNNG (*A*); 100 mg/kg temozolomide (*B*); or 10mg/kg cisplatin (*C*) at time zero. The data show the means of the prevalence of apoptotic bodies per 50 half crypts. Each point represents data from a minimum of three mice. Closed circles, *Msh2* +/+; open circles, *Msh2* −/−. Error bars represent SEM. The difference between +/+ and −/− mice was significant for all three drugs at 6 and 12 hours ($P < 0.05$, Mann–Whitney U test). Significant differences also were seen for temozolomide-treated animals at 24 and 48 hours and for Cisplatin treated mice at 24, 48, and 72 hours.

hours) (Fig. 4*B*). We finally formally excluded the possibility that *Msh2* may have a general role in signaling intestinal apoptosis after DNA damage. Apoptosis of lower crypt cells mediated both by *p53*-dependent and -independent pathways can be initiated by exposure to γ-irradiation (12, 16). However, this apoptosis is quantitatively identical in *Msh2* +/+ and −/− animals (data not shown). Hence, the signal for apoptosis in lower crypt cells injured by methylating agents is specified by recruitment of *Msh2*.

DISCUSSION

These data clearly show that, in response to methylating agent damage, *Msh2* is required for the bulk of both the immediate and delayed apoptotic responses of lower crypt cells. Moreover, although the signal for apoptosis is processed through *p53* where this is available, a death signal is generated in the absence of *p53*. Significantly, in the absence of both genes, no apoptosis was observed. These findings imply that one function of *Msh2* is to mediate the deletion of cells bearing DNA damage of methylation type. It remains possible that the
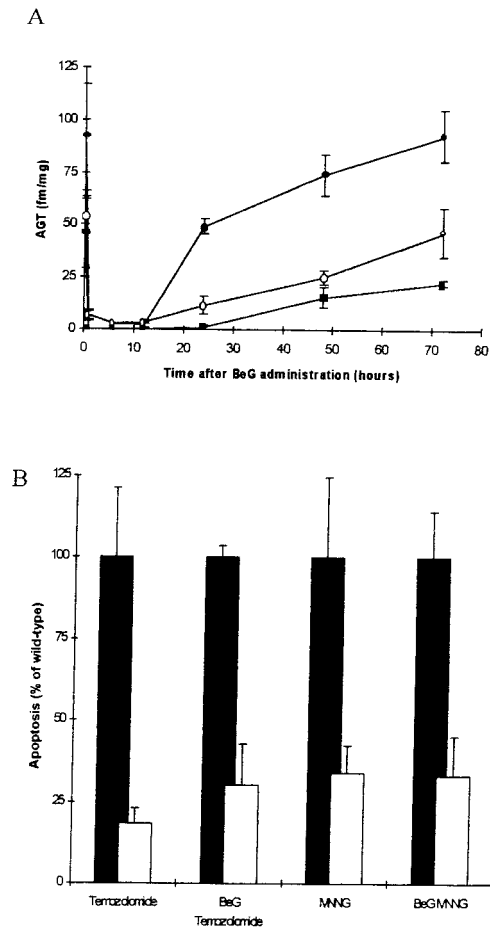
A

B

FIG. 4

A

B

FIG. 3. Influence of AGT on *Msh2*-dependent apoptosis. To competitively deplete AGT function, BeG at a dose of 60 mg/kg body weight was injected i.p. AGT levels were scored in a range of tissues as described (18). (*A*) Sample data is given for the liver (filled circles), colon (open circles), and small intestine (filled squares). In all three tissues, depletion of AGT was seen for the period between 1 and 12 hours after BeG injection. (*B*) One hour after BeG treatment, cohorts of *Msh2* +/+ and −/− mice were treated with 50 mg/kg MNNG and 100 mg/kg temozolomide. Small intestines were harvested 6 hours after drug treatment, and apoptosis was scored. Data are presented showing the percentage reduction in observed apoptosis in the *Msh2* −/− mice (open bars) relative to that in *Msh2* +/+ mice (filled bars standardized to 100%). Non-BeG treated controls are shown for comparison. Each bar represents data from 6 mice. Error bars represent SEM.

FIG. 4. *p53*-dependent apoptosis in the small intestine. (*A*) Mice that were *p53* +/+ or −/− were given i.p. injections of MNNG (50 mg/kg), temozolomide (100 mg/kg), or cisplatin (10 mg/kg) at time zero. The mean prevalence of apoptotic bodies at 6 hours is shown. Each bar represents the data from a minimum of three mice. Closed boxes, *p53* +/+; open boxes *p53* −/−. (*B*) Time course of the levels of apoptosis in *p53* +/+ and −/− mice, and also mice doubly mutant for both *Msh2* and *p53* after injection of temozolomide (100 mg/kg) at time zero (wild-type data reproduced from Fig. 2*B*). Each point represents the data from a minimum of three mice. Closed circle symbols, *p53* +/+; open circle symbols, *p53* −/−; closed triangular symbols, *Msh2* −/− and *p53* −/−. Error bars represent SEM.

cohort of damaged cells protected from apoptosis by the absence of *Msh2* may be removed by death outside the time span analyzed here or that these cells may be prevented from propagating by some other mechanism, such as permanent cell cycle arrest. Nonetheless, the phenomenon of delayed apoptosis that has been observed in *p53* −/− mice after γ-irradiation (12, 16) and seen here after temozolomide was not observed in *Msh2* −/− mice. Hence, the most direct conclusion from this study must be that a significant number of cells escape death by virtue of *Msh2* deficiency and are therefore potential founders of clones bearing unrepaired mutations.

We also have demonstrated that functional *Msh2* suppresses the mutation frequency at the *Dlb-1* locus after *in vivo* exposure to temozolomide. This may arise either through the *Msh2*-dependent deletion of cells we characterize here or as a consequence of *Msh2*-dependent DNA repair. The *in vivo* experiments described here cannot discriminate between these two possibilities; however, *in vitro* data obtained from *Msh2* −/− cells show that, in ES cells, a significant element of the
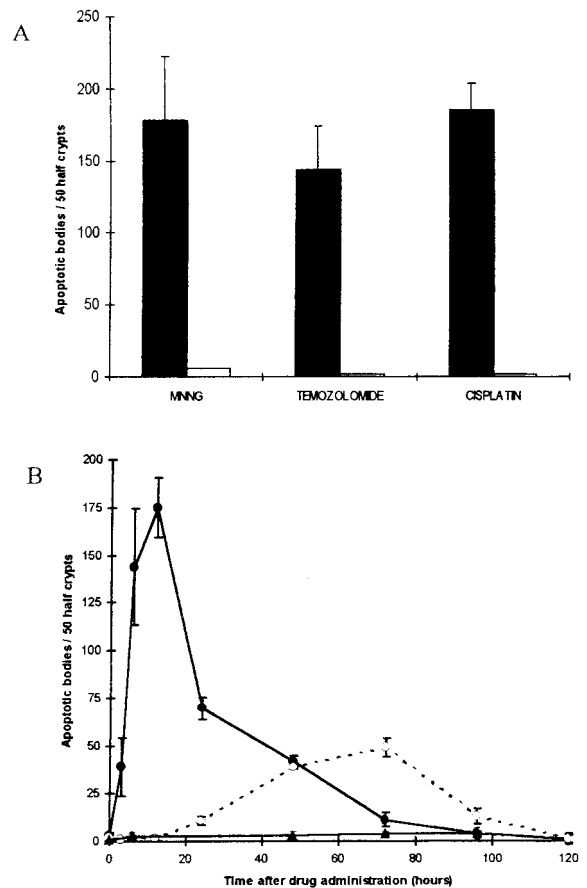
increase in total mutation burden does arise from increased cell survival as assessed by increased clonogenicity. The remaining pertinent question asks, therefore, whether clonogenicity scored in ES cells is directly applicable to clonogenic survival within the intestinal epithelium. An absolute translation of these results to ES cells is questionable for a number of reasons. First, stem cells within the intestine are relatively poorly characterized, and it is probable that ES cells differ from their intestinal counterparts in a number of as yet uncharacterized ways. Second, as discussed above, *in vivo* clonogenic survival of intestinal stem cells is always determined at the level of entire crypts. This makes it impossible to determine the clonogenicity of individual stem cells and also to account for adaptive responses within the crypt, such as the recruitment to the stem cell population of cells normally regarded as external to this compartment. Thus, clonogenicity modeled in ES cells *in vivo* may not directly translate into the intestinal crypt. However, we have demonstrated the principal that, within a stem cell population, clonogenic survival modulates mutation frequency. The hypothesis that this is an important *in vivo* phenomenon now requires testing.

We propose two possible mechanisms to explain the involvement of *p53* in engaging apoptosis. First, it has been proposed that, after methylation, cells die as a result of repeated attempts to correct the mispairs that are generated during new DNA synthesis. Such repeated abortive repair may recruit *p53* and initiate apoptosis, particularly because *p53* is known to bind single stranded DNA (19). Second, *p53* is known to recognize insertion/deletion mismatches (20), and it has been shown that *p53* forms stable complexes at these lesions that may subsequently recruit other proteins. However, we consider this latter mechanism unlikely because *p53* has been shown not to bind GT mismatches.

The finding that administration of the AGT inhibitor BeG does not alter the apoptotic response is surprising in light of experiments that show that AGT inhibition normally sensitizes cells to the effects of alkylation damage (21). This suggests two possibilities: either that lesions other than O^6-methyl-G may trigger *Msh2*-dependent apoptosis or that the drug regimes used here have already functionally depleted AGT function irrespective of the addition of BeG.

In conclusion, we have shown that a significant proportion of the normal *in vivo* apoptotic response of small intestinal cells to two different methylating agents and to the cross-linking agent cisplatin depends on functional *Msh2* and that the degree of this reliance is strongly drug type-dependent. These observations therefore extend previous studies of methylation tolerance to an *in vivo* setting and identify the cellular mechanism of such tolerance. We also show that *Msh2* deficiency leads to a marked increase in the *in vivo* mutation frequency and, furthermore, that, when modeled *in vitro*, a significant element of this rise occurs as a consequence of increased clonogenicity. Given that methylation damage is present in human gastrointestinal DNA (22), these findings raise the possibility that *Msh2* deficiency may predispose to malignancy not only by failing to repair DNA damage but also by failing to engage apoptosis and delete damaged cells in the replicative and stem cell regions of the crypt.

These conclusions raise predictions relevant to tumorigenesis that may now be approached by using mice deficient in *Msh2* and *p53*. For example, an *Msh2*-deficient background should predispose to tumor formation after exposure to temozolomide but not cisplatin. Similarly, *p53* deficiency should show increased predisposition after exposure to both reagents, and deficiency of both genes should lead to a marked sensitivity to temozolomide. Such studies should allow a correlation between the ability to engage cell death and increased tumor predisposition, although the remaining challenge will be to establish the precise contributions of failed cell death and failed DNA repair to this process.

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