Base Excision Repair Intermediates Induce p53-independent Cytotoxic and Genotoxic Responses*

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DNA alkylation damage is primarily repaired by the base excision repair (BER) machinery in mammalian cells. In repair of the N-alkylated purine base lesion, for example, alkyl adenine DNA glycosylase (Aag) recognizes and removes the base, and DNA polymerase β (β-pol) contributes the gap tailoring and DNA synthesis steps. It is the loss of β-pol-mediated 5'-deoxyribose phosphate removal that renders mouse fibroblasts alkylation-hypersensitive. Here we report that the hypersensitivity of β-pol-deficient cells after methyl methanesulfonate-induced alkylation damage is wholly dependent upon glycosylase-mediated initiation of repair, indicating that alkylated base lesions themselves are tolerated in these cells and demonstrate that β-pol protects against accumulation of toxic BER intermediates. Further, we find that these intermediates are initially tolerated in vivo by a second repair pathway, homologous recombination, inducing an increase in sister chromatid exchange events. If left unresolved, these BER intermediates trigger a rapid block in DNA synthesis and cytotoxicity. Surprisingly, both the cytotoxic and genotoxic signals are independent of both the p53 response and mismatch DNA repair pathways, demonstrating that p53 is not required for a functional BER pathway, that the observed damage response is not part of the p53 response network, and that the BER intermediate-induced cytotoxic and genotoxic effects are distinct from the mechanism engaged in response to mismatch repair signaling. These studies demonstrate that, although base damage is repaired by the BER pathway, incomplete BER intermediates are shunted into the homologous recombination pathway, suggesting possible coordination between BER and the recombination machinery.

Cancer, or tumor development, is generally the result of one or more genetic changes in critical growth or cellular maintenance genes. Such changes may be inherited or may occur from environmental exposure (1, 2). In many cases, these genetic changes induce a “mutator” phenotype due to sequence changes in DNA repair or DNA damage checkpoint genes, among others (3). The importance of maintaining the structural and informational integrity of the genome is further highlighted by the plethora of DNA repair and DNA damage checkpoint pathways (2). Consequently, it is important to understand the diverse cellular systems for DNA damage repair available for protection from an enormous array of DNA lesions (4).

The base excision repair (BER)1 pathway is considered the predominant DNA repair system in mammalian cells for eliminating small DNA lesions generated either exogenously or endogenously at DNA bases (4–6). Such DNA damage can be caused by exposure to environmental agents or by normal cellular metabolic processes that produce alkylating molecules, reactive oxygen species, and other reactive metabolites capable of modifying DNA. In the mammalian BER pathway, the damaged base residue is removed by a lesion-specific DNA glycosylase. Subsequently, the resulting abasic site is recognized by apurinic/apyrimidinic endonuclease (APendo), which incises the damaged strand, leaving a single-nucleotide gap with 3'-OH and 5'-deoxyribose phosphate (5'-dRP) groups at the margins. A DNA polymerase β (β-pol)-mediated DNA synthesis step extends from the 3'-OH (7–9), and the 5'-dRP group is removed by the 5'-dRP lyase activity of β-pol (10–14). DNA ligase I or a complex of DNA ligase III and x-ray cross-complementing factor 1 conducts the final, nick sealing, step in the pathway. In addition, several proteins have been observed to form functional partnerships with these BER proteins, including p53, poly(ADP-ribose) polymerase, p300, and proliferating cell nuclear antigen (15–18).

In vivo studies suggested a role for β-pol in varying types of DNA repair (19). Transgenic mice with a homozygous null mutation in the β-pol gene are nonviable after birth, thereby preventing studies on the in vivo function of β-pol (20). However, we utilized β-pol+/− mice to establish embryonic fibroblast (MEF) cell lines homozygous for a null mutation in the β-pol gene (21). β-pol+/− cells are normal in viability and growth characteristics but are more sensitive to nonfunctional alkylating agents such as methyl methanesulfonate (MMS) than wild type cells (21). β-pol possesses both polymerase and 5'-dRP lyase activity.

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1 The abbreviations used are: BER, base excision repair; APendo, apurinic/apyrimidinic endonuclease; β-pol, DNA polymerase β; 5'-dRP, 5'-deoxyribosephosphate; MMS, methyl methanesulfonate; Aag, alkyladenine DNA glycosylase; BrdUrd, bromodeoxyuridine; SCE, sister chromatid exchange; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; 3-Ma, 3-methyladenine; ES, embryonic stem; hAAG, human alkyladenine DNA glycosylase; MEF, mouse embryonic fibroblast; PBS, phosphate-buffered saline; wt, wild type; GFP, green fluorescent protein.
but we found previously that only the 5'-dRP lyase activity of β-pol is essential for resistance to MMS and that β-pol appears to be the major, if not only, protein capable of efficiently removing 5'-dRP groups formed as BER intermediates (22). However, these earlier studies did not determine the source of these repair intermediates, and it was not yet determined if the 5'-dRP groups were formed as a result of BER initiation by a lesion-specific glycosylase. Further, no study to date has begun to unravel the mechanism of cytotoxicity induced by the BER intermediate 5'-dRP.

In this report, we describe the development and characterization of a series of single and double knockout cell lines and mouse models to detail the cytotoxic and genotoxic effects of both alkylated base lesions and the resultant BER intermediate 5'-dRP. We show that 5'-dRP formation (the lethal lesion causing the MMS-sensitive phenotype of β-pol−/− cells) is critically dependent on the alkyladenine DNA glycosylase (Aag), known to remove 3-methyladenine (3-MeA), 7-methylguanine, and other alkylated bases from DNA. Surprisingly, the un repaired N-alkylated purine base lesions are well tolerated in these cells. Interestingly, our results suggest that BER intermediates are associated with an increase in homologous recombination and eventually trigger a block in DNA synthesis. Both the cytotoxic and genotoxic effects in these cells are independent of p53 and the mismatch DNA repair pathway. These studies suggest a possible coordination between BER and the recombination machinery.

EXPERIMENTAL PROCEDURES

Mice and Cells—All breeding was conducted at the NIEHS animal facility following National Institutes of Health, Institutional Animal Care and Use Committee and the Association for Assessment and Accreditation of Laboratory Animal Care approved protocols. The β-pol−/− mice and the Aag−/− mice have been described previously (20, 23). The Apendo−/− mice were generously provided by T. Curran (St. Jude Children’s Research Hospital) (24). The p53−/− mice were generously provided by G. Lozano (M.D. Anderson Cancer Center) (25). The PMS-2−/− mice were generously provided by P. M. Glazer (Yale University) (26). Wild type, β-pol−/−, Aag−/−, β-pol−/−/Aag−/−, p53−/−, β-pol−/−/p53−/−, PMS-2−/−, and β-pol−/−/PMS-2−/− primary MEFs were isolated from 14.5-day-old embryos and, where indicated, transformed by SV40 T-antigen expression, as previously described (21). Genotyping and reverse transcriptase-PCR protocols are available upon request. The MMP-GFP and MMP-hAAG retroviral vectors were developed in conjunction with the Harvard University gene therapy initiative (available on the World Wide Web at hgen.med.harvard.edu/PlasmidRepository.php). MEFs were cultured at 37 °C in a humidified incubator equilibrated with 10% CO2 in DMEM supplemented with 10% fetal bovine serum, penicillin (50 units/ml), streptomycin (50 μg/ml), and Glutamax-I (4 mM) as described (21, 22, 28).

Gap-filling Assay—Whole cell extracts were assayed using a single-nucleoside gap-filling assay. Extracts were incubated in buffer containing 50 mM Hepes, pH 7.8, 2 mM EDTA, 1 mM dithiothreitol, 10 mM MgCl2, 100 nM double-stranded oligonucleotide containing a single uracil (U) residue: 5′-GCC CTG CAG GTC GAU TCT AGA GGA TCC CCG GGT AC-3′. The reaction was incubated for 5 min at 37 °C and analyzed by electrophoresis in a 16% polyacrylamide gel (7M urea, TBE). The reaction was incubated for 5 min at 37 °C and analyzed by electrophoresis in a 16% polyacrylamide gel (7M urea, TBE). Gels were fixed, dried, and autoradiographed.

Alkyladenine glycosylase assay—Aag activity was measured as described previously (23) using a double-stranded oligonucleotide substrate containing a single etheno-adenine (ea) lesion: 29′- (5′-GCC ATC TAT AC CAG TCT ATG AGT GAG TCT ATG C-3′, mouse α-pol mRNA expression. Total RNA was isolated from each cell line and analyzed by reverse transcriptase-PCR for expression of mouse β-pol, mouse Aag, and control mouse actin mRNAs.

Percentage of control growth is as follows: (number of treated cells)/ (number of control cells) × 100.

Flow Cytometric Cell Cycle Analysis—Cell cycle and DNA synthesis was analyzed simultaneously by staining with propidium iodide and incorporation of bromodeoxyuridine (BrdUrd) with some modifications (30). Briefly, cells were seeded in 100-mm dishes at a density of 327,000 cells/dish (equivalent to the cell density used in the cytotoxicity experiments). The following day, cells were treated for 1 h with MMS. At 2 or 8 h after MMS treatment (as indicated), 10 μM BrdUrd (Sigma) was added to the dishes for 30 min to pulse-label the cells. Cells were then washed with PBS, harvested by trypsinization, and washed a second time with PBS. The cell pellet obtained after centrifugation was resuspended in 100 μl of cold PBS, and the cells were dropped slowly into 70% ethanol and allowed to fix at 4 °C overnight. The samples were washed, suspended in 2 N HCl containing 0.5% Triton X-100, and incubated for 30 min at room temperature to denature the DNA. The cells were pelleted, washed with PBS, and resuspended in 1 ml of PBS containing 5 μg/ml propidium iodide (Sigma). The samples were analyzed by flow cytometry using Cell Quest software (BD Biosciences). Cell cycle populations are designated G0/G1 (2 N DNA content with no BrdUrd incorporation), S (variable DNA content with BrdUrd incorporation), and G2/M (4 N DNA content without BrdUrd incorporation).

Sister Chromatid Exchange Assay—For sister chromatid exchange (SCE) measurements, 1 × 106 cells were seeded onto 75-cm2 tissue culture dishes 8 h before drug treatment. The cells were treated with MMS in complete McCoy’s 5A medium (Invitrogen) supplemented with 10 μM BrdUrd for 1 h at 37 °C in 5% CO2. Following drug treatment, the cells were incubated in McCoy’s 5A medium supplemented with 10 μM BrdUrd for 20 h, Coomassie (0.1 μg/ml; Invitrogen) was included for the last 2 h of incubation, and the cells were subsequently harvested by mitotic shake-off, resuspended, and incubated for 15 min at 37 °C in hypotonic solution (0.2% potassium chloride, 0.2% sodium citrate, and 10% fetal bovine serum) and then fixed in Carnoy’s solution. To produce “harlequin” chro-
mosomes, a modified fluorescence plus Giemsa technique was used (31). Slides were stained in Hoechst 33258 (5 μg/ml) for 20 min, mounted in 0.067 M Sorensen’s buffer with a coverslip, and exposed to a General Electric 15-watt black light bulb at 65 °C for 20 min. Slides were then heated at 65 °C in 20× SSC for 20 min, rinsed, and stained in a 5% Giesma solution in 0.067 M Sorensen’s buffer. Twenty second-division metaphase spreads were counted per data point.

RESULTS

Aag Is Required to Initiate BER of MMS- and MNNG-induced Lesions to Generate the Toxic 5′-dRP Repair Intermediate—To study the cytotoxicity and genotoxicity of both alkylated DNA bases and BER repair intermediates, we developed a set of isogenic MEF cell lines with homozygous null mutations
in the Aag gene, in the β-pol gene or in both the Aag and β-pol genes (Fig. 1). To ensure that no significant backup or compensatory enzymatic activity was present, extracts from each were analyzed for Aag-specific activity (Fig. 2a) and β-pol-specific BER gap-filling activity (Fig. 2b). Wild type and β-pol−/− cells showed the expected levels of Aag activity, with little or no Aag-specific activity in the Aag−/− and β-pol−/−/Aag−/− cells (Fig. 2a), whereas each had similar levels of uracil-DNA glycosylase activity (data not shown). Conversely, the wild type and Aag−/− cells reported robust β-pol-specific BER activity with little or no activity seen in extracts from β-pol−/− or β-pol−/−/Aag−/− cells (Fig. 2b).

The cytotoxicity of methylating agent-induced DNA base damage is increased in both Aag−/−/ embryonic stem (ES) cells (32) and β-pol−/− MEFs (21), implying that both methylated bases and BER intermediates are cytotoxic. We therefore compared the cytotoxicity of MMS in wild type and isogenic β-pol−/− (Fig. 2c) and Aag−/− MEFs (Fig. 2d). Consistent with the known phenotype of these cells and the hypothesis that the 5′-dRP lyase activity of β-pol is essential for reversing MMS-induced cytotoxicity (22), β-pol−/− MEFs were more sensitive to MMS than wild type MEFs, as expected (Fig. 2c).

Sensitivity of fibroblast cell lines to MNNG. Shown are survival curves in the presence of MNNG of wild type cells (filled circles) versus β-pol−/− cells (filled triangles) (a), wild type cells (filled circles) versus Aag−/− cells (filled squares) (b), and β-pol−/− cells (filled triangles) versus β-pol−/−/Aag−/− cells (filled diamonds) (c). The arrow indicates the shift of the curve due to the genetic loss of β-pol (a) or the genetic loss of Aag (c).
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**FIG. 4.** Percentage of cells incorporating BrdUrd after exposure to MMS. Wild type cells, β-pol<sup>+/−</sup> cells, Aag<sup>−/−</sup> cells, and β-pol<sup>−/−</sup>Aag<sup>−/−</sup> cells were treated with 1 mM MMS for 1 h, and the percentage of BrdUrd-positive cells at 2 and 8 h following initiation of MMS treatment was evaluated following a 30-min pulse with BrdUrd, as described under “Experimental Procedures.” a, a summary of the experiments; b, a representative fluorescence-activated cell sorting analysis of β-pol<sup>−/−</sup> cells as indicated. c, depicts a representative fluorescence-activated cell sorting analysis of β-pol<sup>−/−</sup>Aag<sup>−/−</sup> cells as indicated.

**TABLE I**

| No. of male | No. of female | No. of male | No. of female | No. of male | No. of female |
|-------------|---------------|-------------|---------------|-------------|---------------|
| Aag<sup>−/−</sup> | Aag<sup>−/−</sup> | Aag<sup>−/−</sup> | Aag<sup>−/−</sup> | Aag<sup>−/−</sup> | Aag<sup>−/−</sup> |
| Expt. 1<sup>a</sup> | | | | | |
| Totals | 12 | 12 | 18 | 25 | 0 | 0 |
| Percentages | 17.9 | 17.9 | 26.8 | 37.3 | 0 | 0 |
| Expt. 1 | | | | | |
| No. of Aag<sup>−/−</sup> | No. of Aag<sup>−/−</sup> | No. of Aag<sup>−/−</sup> | No. of Aag<sup>−/−</sup> | No. of Aag<sup>−/−</sup> | No. of Aag<sup>−/−</sup> |
| Expt. 2<sup>b</sup> | | | | | |
| Totals | 24 | 43 | 0 | |
| Percentages | 35.8 | 64.2 | 0 | |
| Expt. 2 | | | | | |
| No. of β-pol<sup>−/−</sup>Aag<sup>−/−</sup> | No. of β-pol<sup>−/−</sup>Aag<sup>−/−</sup> | No. of β-pol<sup>−/−</sup>Aag<sup>−/−</sup> | No. of β-pol<sup>−/−</sup>Aag<sup>−/−</sup> | No. of β-pol<sup>−/−</sup>Aag<sup>−/−</sup> | No. of β-pol<sup>−/−</sup>Aag<sup>−/−</sup> |
| | | | | | |
| a 10 litters with a total of 67 weanlings, 21 days old. b 22 litters with a total of 135 weanlings, 21 days old.

**Aag Deficiency Does Not Rescue the Embryonic or Perinatal Lethality of the Aag<sup>−/−</sup> and the β-pol<sup>−/−</sup> Mutation in Mice**—The cellular phenotype associated with a genetic loss of β-pol (i.e. a DNA damage-induced accumulation and cytotoxicity of the 5’-dRP lesion) (21, 22), raised the question of whether the lethality of the β-pol<sup>−/−</sup> mutation in mice (20, 35, 36) is
The BER Intermediate 5′-dRP Induces a Rapid and Severe Block to Replication—MMS has long been used to study DNA damage-induced cell cycle checkpoints and inhibition of DNA replication (39, 40). For example, in recent studies with wild type strains of budding yeast, MMS-induced DNA damage was shown to reduce the rate of replication fork progression (39). By examining the effect of MMS on S-phase-associated DNA synthesis in the isogenic cell lines described above, we could dissect the impact of MMS-induced base damage versus 5′-dRP BER intermediates on DNA replication. Using a combination of BrdUrd incorporation, propidium iodide staining, and flow cytometry, we discovered that both MMS-induced base damage (accumulated in Aag<sup>−/−</sup> and β-pol<sup>−/−</sup>/Aag<sup>−/−</sup> cells) and MMS-induced BER intermediates (accumulated in β-pol<sup>−/−</sup> cells) blocked DNA synthesis (Fig. 4). However, the inhibition was both modest and transient in the Aag<sup>−/−</sup> and β-pol<sup>−/−</sup>/Aag<sup>−/−</sup> cells compared with the β-pol<sup>−/−</sup> cells, where the block was both severe and persistent. Fig. 4 shows that 3-MeA (plus other Aag substrates) presents a mild replication block visible at 2 h after MMS exposure but that synthesis fully recovers by 8 h. In contrast, 5′-dRP BER intermediates severely block replication by 2 h, and the block is almost complete by 8 h. Thus, it would seem that the alkylation bases acted upon by Aag can ultimately be tolerated or avoided in these cells, presumably by the action of mechanisms such as DNA lesion bypass or recombination (39, 40); such avoidance would account for the fact that 3-MeA lesions are not toxic to these cells (Figs. 2–4). Apparently, 5′-dRP lesions are not tolerated in these cells and thus block replication and lead to cell death (Figs. 2–4).

Cytotoxicity and Genotoxicity of 5′-dRP Is Independent of p53 and the Mismatch DNA Repair Pathway—A hallmark response to DNA damage is the activation of the tumor suppressor gene p53 and the subsequent induction of the p53 network (30, 41–43). Stabilized p53 participates in either halting cell cycle progression to allow completion of DNA repair (30, 41–43) or activating apoptotic programmed cell death (30, 41–43). Stabilized p53 participates in either halting cell cycle progression to allow completion of DNA repair (30, 41–43) or activating apoptotic programmed cell death (30, 41–43). Stabilized p53 participates in either halting cell cycle progression to allow completion of DNA repair (30, 41–43) or activating apoptotic programmed cell death (30, 41–43). However, the results
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Fig. 7. Evidence that β-pol substrates induce recombination. Average number of sister chromatid exchanges per chromosome were determined as described under “Experimental Procedures” for each cell line following exposure to MMS or medium control. a, wild type cells (filled circles), β-pol−/− cells (filled triangles), Aag−/− cells (filled squares), and β-pol−/−/Aag−/− cells (filled diamonds). b, wild type (+hAAG) cells (open circles), β-pol−/−/+hAAG cells (open triangles); Aag−/−/+hAAG cells (open squares), and β-pol−/−/Aag−/−/+hAAG cells (open diamonds). Twenty second-division metaphase spreads were counted per data point.

shown here indicate that whereas p53 may stimulate BER, it is not required for BER function. In addition, we have previously demonstrated that the mismatch DNA repair pathway recognizes alkylated bases (O6-MeG) and that such recognition initiates an apoptotic response (45). We developed mismatch DNA repair-deficient MEFs (PMS-2−/−) and β-pol−/−/PMS-2−/− cells) to determine whether the 5′-dRP-induced cytotoxicity is related to this mismatch repair-dependent apoptotic repair mechanism. As shown in Fig. 6, the MMS-mediated hypersensitivity of β-pol−/− cells is not altered by a deficiency in the mismatch repair gene PMS-2 (Fig. 6).

5′-dRP Lesions Are Initially Tolerated by Homologous Recombination—DNA damage-induced arrest of the replication fork may be overcome by homologous recombination mechanisms of lesion avoidance (46, 47). Since β-pol substrates or BER intermediates (5′-dRP) block replication (Fig. 4), we determined whether these BER intermediates can also induce recombination. At minimally toxic doses of MMS (up to 0.2 mM), we compared the genotoxic effect of both methylated base damage and BER intermediates by measuring the induction of SCEs. As shown in Figs. 7 and 8, MMS treatment of wild type, Aag−/−, and β-pol−/−/Aag−/− MEFs generated a similar dose-dependent increase in SCE events. However, MMS-induced BER intermediates (accumulating in β-pol−/− cells) generated relatively more SCE events, indicating that 5′-dRP lesions can indeed stimulate homologous recombination. Similar to the pattern seen in the cytotoxicity assays, β-pol−/− cells require Aag activity to give rise to the higher than wild type levels of MMS-induced SCE events (Fig. 7).

In the absence of Aag, compensatory pathways (alternative DNA glycosylases or the nucleotide excision repair pathway) (27, 34) did not effectively remove MMS-induced or MNNG-induced alkylation damage. Accumulated alkylation damage then slows progression of the replication fork in a checkpoint-independent fashion (40). The studies described here, together with our earlier report on MMS-induced mutagenesis (28), support a model whereby a mutagenic lesion bypass mechanism allows for survival after alkylation damage, and this lesion tolerance mechanism is independent of p53. Conversely, the cytotoxicity of 3-MeA is probably a p53-dependent effect (33). However, once BER is initiated, damage-specific glycosy-
lates give rise to accumulation of β-pol substrates (5'-dRP). 5'-dRP causes a complete block to replication fork progression and possibly induces fork regression and resolution by BLM and WRN (40, 46). β-pol deficiency therefore results in an increase in damage-induced recombination and toxicity in a p53-independent and mismatch DNA repair-independent mechanism. The 5'-dRP-mediated cytotoxicity described herein may well represent a novel mechanism of checkpoint activation and replication arrest. Finally, it remains to be determined whether the presence of p53 will alter this DNA damage response paradigm and, further, whether BER and homologous recombination repair complexes, in a coordinated effort, facilitate survival following base damage.

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![Figure 8. Genotoxicity of MMS as determined by SCE analysis. Shown are representative chromosome spreads of β-pol [−−] cells untreated (a), β-pol [−−] cells treated with 0.2 mM MMS for 1 h (b); β-pol [−−]+[hAAG] cells untreated (c), and β-pol [−−]+[hAAG] cells treated with 0.2 mM MMS for 1 h (d). In each panel, the arrow points to an example of SCE event.](image-url)
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