3-Methyladenine DNA Glycosylase-deficient Aag Null Mice Display Unexpected Bone Marrow Alkylation Resistance

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Abstract

Most cells deficient in 3-methyladenine (3MeA) DNA glycosylase become sensitive to the lethal and clastogenic effects of alkylating agents. Surprisingly, myeloid progenitor bone marrow (BM) cells derived from Aag−/− mice were more resistant than those from wild-type mice to the cytotoxic effects of several alkylating agents. Moreover, an alkylation-resistant phenotype was observed in vivo using the BM micromenous assay as a measure of chromosome damage. Flow cytometry indicated that in vivo alkylation resistance in Aag null BM cells may be restricted to cells of the myeloid lineage. These results show that in particular cell types, the initiation of base excision repair is more lethal to the cell than leaving the damaged bases unrepaired by Aag.

Introduction

All organisms are constantly exposed to alkylating agents, not only from our exogenous environment but also from normal cellular metabolites. The many nucleophilic sites of DNA are readily modified by electrophilic alkylating agents, generating alkylated DNA lesions in the process. Among these alkylated lesions, N7-alkylguanines are usually considered to be innocuous, whereas N2-alkyladenines and O6-alkylguanines are known to be both cytotoxic and mutagenic. Alkylated DNA bases are primarily recognized and repaired via the actions of two kinds of repair proteins, O6-alkylguanine DNA Mtransferases and 3MeA DNA glycosylases. Mtransferases directly remove alkyl groups from O6-alkylguanines (and to a lesser extent from O6-alkylthymines), toxic lesions generated by most chemotherapeutic alkylating agents. Numerous studies show that Mtransferase-deficient Escherichia coli, Saccharomyces cerevisiae, and mammalian cells are very sensitive to the mutagenic and cytotoxic effects of chemical alkylating agents (1, 2). 3MeA DNA glycosylases, encoded by AAGIMP/GANPG in humans and Aag/Mpg/Apg in mice, initiate the BER pathway (3–5). In addition to excising 3MeA, these glycosylases excise DNA bases with methyl or certain other alkyl groups at the N7 and N3 positions of both adenine and guanine; they also excise eA and deaminated adenine (hypoxanthine) DNA bases (1, 6). Aag-initiated BER begins with cleavage of the glycosidic bond connecting the modified base to the deoxyribose sugar; the resulting abasic site can then be recognized by AP endonucleases that cleave the phosphodiester bond 5′ to the abasic site forming a strand break with a 3′ OH and a 5′ deoxyribose phosphate terminus. DNA polymerase β can extend from the 3′ OH terminus. In addition, polymerase β can remove the 5′ deoxyribose phosphate residue via an enzymatic activity residing in its NH2-terminal domain. BER is completed by either DNA ligase I or a DNA ligase III/XRCCI complex that joins the two resulting ends, restoring the original, unmodified DNA structure (1). It is important to note that each of the intermediates generated during the BER process represents some form of DNA damage and that it is not until DNA ligation is completed that the genome is free from damage. An appreciation of this fact is important for understanding why some surprising and unexpected phenotypes are produced when BER enzymes are inappropriately expressed. Here we show that decreased expression of the Aag 3MeA DNA glycosylase confers unexpected alkylation resistance on some mouse tissues, rather than the expected alkylation sensitivity.

Materials and Methods

Generation of Crude Protein Extracts from Mouse Tissues. C57Bl/6J 8-week-old mice were euthanized by CO2 asphyxiation, and several tissues were immediately harvested, frozen in liquid nitrogen and then processed to generate crude protein extracts (7). In addition, DNA content was determined for each crude sonicate (8).

In Vitro Oligonucleotide DNA Glycosylase Assay. A double-stranded 25mer oligonucleotide containing a single centrally located eA residue [5′-GGATCATCGTTTTTC(eA)GCTACATCGC-3′] was used as template DNA. Glycosylase assays were performed according to Engelward et al. (7). A phosphorimager was used both to visualize and to quantitate Aag DNA glycosylase activity.

BM Cell Clonogenic Survival Assay. BM cells were harvested from the femurs of Aag+/+ and Aag−/− mice and then treated with MMS, MeLex, TMZ, MMC, BCNU, and thiotepa. Cells were then washed and resuspended in complete medium, mixed with methylcellulose (Stem Cell Technologies, Vancouver, British Columbia, Canada), and plated in duplicate. After 10–14 days, colonies of >50 cells were scored, and percentage survival was determined by comparing treated and untreated duplicate cultures processed similarly.

Mimicronucleus Assay. Wild-type and Aag null mice received i.p. injections of PBS, MMS, MMC, TMZ, and BCNU. Twenty-four h later, BM cells were harvested and processed for analysis (9). PCEs, MN PCEs, and NCEs were then scored using a Nikon Eclipse E800 fluorescent microscope with a ×60 objective. PCEs (2000/mouse) were scored for the presence of micronuclei. In all of the experiments performed, the ratio of PCEs/NCEs was between 40 and 60%, which indicated that the dose of the alkylating agent used was subtoxic.

Flow Cytometry to Determine MMS Sensitivity of Lineage-specific BM Cells. Mice received i.p. injections of PBS or MMS, and 24 h later, BM cells were harvested, washed, and resuspended in HBSS to 1 × 107 cells/ml. For each sample, six 100-μl aliquots were treated with either phycoerythrin-antimouse CD3 (T-cell specific), phycoerythrin-antimouse CD116 (Mac1, macrophage and granulocyte-specific), FITC-antimouse Ly-6G (Gr1, granulocyte-specific), FITC-antimouse CD45R/B220 (B-cell-specific), or IgG isotype nonspecific control antibody (BD PharMingen). A Coulter Elite flow
cytometer was used to determine the fraction of each BM cell lineage present before and after alkylating agent treatment.

30-Day Survival Studies. Aag +/- and Aag –/– mice received i.p. injections of MMS and MMC. A dose schedule was followed as recommended by Deichmann and LeBlanc (10); one mouse/dose received an injection and six Aag +/- and Aag –/– mice/drug/experiment received an injection with each dose being 50% greater than the preceding dose. The lowest dose at which a mouse dies within 30 days has been shown empirically to be a good estimate of the LD₅₀ (10).

Results

BM Cells Express Low Levels of 3MeA DNA Glycosylase. BM cells, particularly of the myeloid lineage, are acutely sensitive to alkylating agent cytotoxicity. A plausible explanation for such sensitivity is that, relative to other cell types, these cells are deficient in activities that recognize and repair DNA alkylation damage. Indeed, Gerson et al. (11) found that both mouse and human BM cells express very low levels of the Mgmt O⁶-methylguanine DNA MTase relative to other tissues. Here, we set out to determine whether Aag 3MeA DNA glycosylase levels are similarly low by comparing Aag DNA glycosylase activities in mouse BM, spleen, liver, lung, kidney, brain, and gastrointestinal tract tissues. The activity of this glycosylase was determined by an in vitro assay that measures the production of alkali labile abasic sites on release of εA bases from an oligonucleotide substrate (see “Materials and Methods”). As depicted in Fig. 1, BM cells expressed the lowest Aag activity among the tissues analyzed, with the exception of the gastrointestinal tract. Liver and brain showed the highest levels of glycosylase activity followed by (in descending order) the kidney, lung, and spleen. [It should be noted that we previously determined that all of the detectable εA DNA glycosylase activity in mouse tissue extracts is attributable to the presence of Aag (7)]. These data suggest that BM cells may be limited in their ability to excise alkylated DNA bases and, more importantly, that this limitation may contribute to their extreme sensitivity to alkylation-induced cytotoxicity and clastogenicity. However, it is important to point out that for the analogous Mgmt MTase deficiency in BM cells, the residual very low level of MTase provides surprisingly extensive alkylation protection, as evidenced by the finding that Mgmt –/– BM cells of the myeloid lineage are much more sensitive than wild-type BM cells to a number of alkylating agents (12). It seemed to us that this could also hold true for the Aag DNA glycosylase, i.e., that even very low Aag levels might play an important role in protecting BM cells against alkylation toxicity. We, therefore, set out to compare the alkylation sensitivity of BM cells derived from Aag –/– and Aag +/- mice.

Aag Null BM Cells Are Resistant to Alkylation-induced Cytotoxicity. Clonogenic survival assays were performed with BM cells harvested from Aag +/- and Aag –/– mice. BM cells were treated ex vivo with MMS, MeLex, BCNU, TMZ, MMC, or thiotepa and then plated on semisolid media to allow myeloid lineage hematopoietic progenitor survival assays were performed to determine the sensitivity of BM cells derived from Aag +/- (□) and Aag –/– (●) mice to: (A) methylmethane sulfonate (MMS), (B) methyl-lexitropsin (MeLex); (C) TMZ; (D) BCNU; (E) MMC, and (F) thiotepa. Experiments were done a minimum of three times each; representative results are shown.

Fig. 2. Ex vivo alkylation sensitivity of BM cells from Aag null and wild-type mice. Hematopoietic progenitor survival assays were performed to determine the sensitivity of BM cells from Aag +/- (□) and Aag –/– (●) mice to: (A) methylmethane sulfonate (MMS), (B) methyl-lexitropsin (MeLex); (C) TMZ; (D) BCNU; (E) MMC, and (F) thiotepa. Experiments were done a minimum of three times each; representative results are shown.
**Aag Null BM Cells Display In Vivo Alkylation Resistance.** The micronucleus test is commonly used to assess large-scale chromosome damage in mammalian cells after in vivo treatment with a DNA damaging agent. In this study, Aag +/+ and Aag −/− mice received i.p. injections of MMS, MMC, TMZ, BCNU, or a vehicle control, 24 h before harvesting BM cells to monitor the generation of MN PCEs that reflects the extent of clastogenicity. Aag null cells were more resistant than wild-type cells to the clastogenic effects of MMS and MMC because both MMS and MMC induced significantly more chromosomal damage in the repair proficient Aag +/+ mice versus the repair deficient Aag −/− mice (Fig. 3). In contrast, Aag status had no influence on in vivo clastogenicity after BCNU and TMZ exposure. Thus, from both the ex vivo hematopoietic progenitor survival assays and the in vivo micronucleus assay in PCEs, our results clearly indicate that Aag null BM cells are more resistant than wild-type cells to the lethal and clastogenic effects of the alkylating agent MMS. However, for MMC and TMZ the two assays did not concur, in that Aag status influenced clastogenicity but not cytotoxicity for MMC, and influenced cytotoxicity but not clastogenicity for TMZ. We believe these complex results reflect the fact that each assay monitors damage sensitivity in different cell types and, moreover, that individual cell types display complex phenotypic differences.

**BM Cell Resistance to Alkylating Agents in Aag Null Animals Is Lineage-specific.** In Fig. 2 and 3, we showed that Aag expression renders hematopoietic progenitors of the myeloid lineage, as well as PCEs (also of the myeloid lineage), sensitive to the toxic effects of MMS. The BM compartment is composed of many different cell types and lineages, and we reasoned that one might express different 3MeA DNA glycylase levels (as well as different levels of other repair enzymes); as a result each lineage may show differences in their MMS sensitive phenotype. To test this, BM was harvested from Aag null and wild-type mice 24 h after i.p. injection with MMS (30 mg/kg) or vehicle and analyzed for the remaining levels of different cell lineages. In vehicle-treated Aag null and wild-type mice the distribution of granulocytes, macrophages, T, or B lymphocytes, was not significantly different (data not shown). However, after MMS exposure, BM cells with granulocyte and macrophage lineage markers were significantly depleted in Aag-expressing wild-type mice relative to Aag null mice. In contrast, cells staining positive for B- or T-lymphocyte markers were equally represented in both lineages of mice (data not shown). These finding indicates that 3MeA DNA glycosylase status modulates MMS sensitivity in a lineage specific fashion, and that the action of the glycylase on MMS-induced DNA damage produces a toxic effect in the myeloid (granulocyte and macrophage), but not the lymphoid (B and T lymphocyte) lineages.

**The Aag Phenotype Has No Influence on Alkylating Agent-induced Animal Lethality.** To determine whether 3MeA DNA glycosylase expression influences animal lethality after alkylating agent treatment, 30-day survival assays were performed to estimate LD50 values for MMS in wild-type and Aag null mice. Engelward et al. (14) and Allan et al. (15) had previously shown that Aag null ES cells are sensitive to the cytotoxic and clastogenic effects of MMS. On the basis of these findings one might expect Aag null animals to be MMS sensitive. Alternatively, based on the alkylination resistance of Aag null BM cells versus wild-type BM cells described in our current report, one might expect Aag null animals to be MMS resistant. In fact, neither expectation turned out to be correct. The LD50 experiments indicated no significant differences in MMS-induced lethality between wild-type and Aag null mice, with an approximate LD50 value of 150 mg/kg (data not shown). Thus, Aag 3MeA DNA glycylase status does not appear to modulate lethality to MMS in the whole animal, although for individual cell types, the presence or absence of this DNA repair activity can have a profound effect on MMS-induced toxicity. Whether Aag status modulates alkylating-induced tumorigenesis is under investigation.

**Discussion**

The repair of potentially cytotoxic O6-alkylguanine DNA lesions is a one-step direct reversal process that does not involve the cleavage of any covalent bonds in DNA other than that connecting the unwanted alkyl group to the O6 position of guanine. In contrast, all excision repair processes require multiple steps involving the cleavage of several covalent bonds at sites flanking the DNA damage, followed by new DNA synthesis and DNA ligation. For BER, this means the generation of several types of damaged DNA as intermediates in the repair process. Given that each intermediate, if left unrepaired, can itself be toxic to the cell, it is not surprising that producing an imbalance between BER enzymes might lead to increased mutation or cell death. Indeed, a growing body of evidence, using bacterial and yeast model systems, has recently emerged to support this notion (16, 17). Here we demonstrate that imbalances can produce changes in alkylating sensitivity in an animal, namely the mouse, and more importantly, that the changes can be either positive or negative (or neutral) depending on the specific cell and tissue type.

Initial studies showed that Aag −/− mouse ES cells are more sensitive than wild-type cells to the chromosome damage and cell killing that follows alkylating treatment (14, 15). This observation was not surprising because it was analogous to the earlier characterization of 3MeA DNA glycosylase deficient E. coli and *S. cerevisiae* model organisms, and because it was consistent with 3MeA lesions blocking DNA replication (1, 18). Elder et al. (19) also found that Aag −/− mouse PEFs are more sensitive to MMS-induced cytotoxicity, and that Aag −/− T lymphocytes are more sensitive to MMS-induced mutation. Taking all these results together, our present observation that certain Aag −/− BM cells are more resistant to alkylating agent-induced cytotoxicity and clastogenicity than their Aag +/+ counterparts was quite unexpected. However, some of our more recent studies using *S. cerevisiae, Schizosaccharomyces pombe* and *E. coli* as model organisms for studying BER might provide a framework for understanding how the same genotypic change in mouse ES, PEF, and BM cells could produce such different phenotypes. A brief summary of these studies follows.

In *S. cerevisiae*, overexpression of the Mag1 3MeA DNA glyco-
cosylase increases spontaneous mutation and sensitizes cells to MMS-induced cytotoxicity (12, 18). This phenotype was largely suppressed by the cooverexpression of an AP endonuclease, which indicated that it is a glycosylase/AP endonuclease imbalance that produces the mutator and alkylation sensitive phenotypes, presumably by leading to the accumulation of mutagenic and cytotoxic abasic sites in the yeast genome (12). Posnick and Samson (17) showed that overexpression of 3MeA DNA glycosylases in E. coli similarly increased spontaneous mutation and MMS-sensitivity. It is therefore clear that improper balance, and thus improper coordination, between consecutive BER steps can be detrimental to the cell. Indeed, biochemical evidence now supports the notion that the individual steps of BER are coordinated via specific protein-protein and protein-DNA interactions (20, 21). In addition, we recently demonstrated unexpected cross-talk between BER and other DNA repair pathways. Memisoglu and Samson (22) knocked out the major 3MeA DNA glycosylase in three S. pombe strains with different repair capacities, and this resulted in three different phenotypes. In wild-type cells, glycosylase deficiency had virtually no effect on MMS-sensitivity, indicating that BER normally plays a minor role in 3MeA repair in this organism; other experiments indicated that NER and homologous recombination repair play a much more predominant role in preventing the toxicity induced by 3MeA. In NER-deficient cells, the elimination of 3 MeA DNA glycosylase rendered cells MMS-sensitive, which indicated that BER can play a back-up role when NER is absent. However, elimination of the same glycosylase in recombination-deficient cells rendered them MMS resistant. We infer from this surprising result that one or more of the intermediate steps in BER is limiting in S. pombe and that the homologous recombination repair pathway normally processes the strand breaks that would accumulate under these conditions. Thus, in the absence of recombination the cell actually survives better if BER is not initiated at 3MeA lesions, because 3MeAs are presumably less toxic to the cell than unrepaird strand breaks. The replication blocking 3MeA lesions may be relatively less toxic than DNA strand breaks for one or both of the following reasons: (a) NER can complete the repair process at 3MeAs; (b) 3MeAs can be bypassed by translesion DNA polymerases (23). These results using microbial cells may help provide hypotheses as to why mouse ES, PEF, and BM cells show different alkylation-sensitive phenotypes when the Aag 3MeA DNA glycosylase is eliminated.

Aag-elimination renders ES and PEF cells MMS sensitive (14, 15), indicating that Aag activity protects against alkylation-induced cell death. Presumably, in these cells, BER, initiated by wild-type levels of Aag, can be properly completed to a ligated DNA product, and such completion may be achieved via the downstream BER enzymes or via homologous recombination at BER intermediates. However, in the myeloid BM cells it is possible that Aag-initiated BER does not always go to completion, either by BER or by recombination, resulting in longer-lived BER intermediates, and thus longer-lived DNA strand breaks. Because unrepaird DNA strand breaks are known to be cytotoxic, and are likely to be more toxic than unrepaired alkylated bases, Aag-elimination would render such cells MMS-resistant. Indeed, this may explain why the BM tissue has evolved to express such low levels of the Aag 3MeA DNA glycosylase. One prediction from this model that is currently being tested, is that the introduction of extra 3MeA DNA glycosylase activity via gene transfer into BM stem cells would render BM more alkylation sensitive than wild-type BM. This possibility has significant implications for the design of gene therapy vectors for conferring alkylation resistance to the BM of patients undergoing cancer chemotherapy.

One in three people in the developed world will be diagnosed with cancer at some point during their lifetime, a significant fraction of whom will undergo alkylation agent chemotherapy. It is, therefore, inevitable that large numbers of individuals in our society will be deliberately exposed to alkylation agents despite the inevitable myelosuppressive side-effects of such agents (24, 25). For this reason, we seek to understand exactly how BM cells can be protected from alkylation-induced toxicity. In this regard, it is notable that, on their characterization, two DNA alkylation repair-deficient mouse models have each produced surprising results. Initially, it seemed logical that low Mgmt and Aag levels in BM were primarily responsible for the alkylation sensitivity of this tissue. However, it turns out that the low level of Mgmt plays an unexpectedly profound role in protecting BM against alkylation agents (12, 26), and that the low level of Aag in BM cells plays an unexpected negative role in protecting against alkylation agents. We surmise that before we can understand exactly how cells are protected against alkylation damage, it is important to understand how these repair activities integrate with other repair activities being expressed in the cell, and further, to understand how DNA repair overall integrates with other determinants of alkylation-induced toxicity.

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References


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