Both base excision repair and O6-methylguanine-DNA methyltransferase protect against methylation-induced colon carcinogenesis

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Methylating agents are widely distributed environmental carcinogens. Moreover, they are being used in cancer chemotherapy. The primary target of methylating agents is DNA, and therefore, DNA repair is the first-line barrier in defense against their toxic and carcinogenic effects. Methylating agents induce in the DNA O6-methylguanine (O6MeG) and methylations of the ring nitrogens of purines. The lesions are repaired by O6-methylguanine-DNA methyltransferase (Mgmt) and by enzymes of the base excision repair (BER) pathway, respectively. Whereas O6MeG is well established as a pre-carcinogenic lesion, little is known about the carcinogenic potency of base N-alkylation products such as N3-methyladenine and N3-methylguanine. To determine their role in cancer formation and the role of BER in cancer protection, we checked the response of mice with a targeted gene disruption of the BER initiated by Aag methyltransferase (Mgmt), which repairs O6MeG in a single-step suicide reaction (9), is highly efficient in suppressing point mutations and genotoxicity in vitro and in vivo (for review see ref. 10). Mgmt was also shown to prevent from cancer formation induced by O6MeG-producing agents. Thus, human Mgmt expressed in mice reduced N-methyl-N-nitrosourea (MNU)-induced thymomas (11) and liver tumors upon dimethylnitrosamine exposure (12). It also protected against lung carcinogenesis (13) and azoxymethane (AOM)-induced aberrant crypt foci and mutations in K-ras (14). Mice expressing human Mgmt in skin were protected from skin tumor formation induced by MNU and the chloroethylating anticancer drug ACNU (nimustine), using the two-stage tumor initiation–promotion protocol in which 12-O-tetradecanoylphorbol 13-acetate was applied as tumor promoter (15,16). Mgmt transgenic overexpression also protected against MNU-induced conversion of benign into malignant tumors (17). In contrast, Mgmt-lacking mice are more sensitive than isogenic wild-type (WT) mice to the genotoxic effects of methylating agents (18–20). They are also highly vulnerable to cancer induction by alkylating agents, which was shown for the formation of thymic lymphomas (21) and colonic aberrant crypt foci (22).

O6MeG is not only a pre-mutagenic and pre-carcinogenic but also a pre-cytotoxic DNA lesion. Toxicity triggered by O6MeG is dependent on the processing of O6MeG/thymine mispairs by MutSx-dependent mismatch repair, in which thymine is excised and then reinserted opposite the O6MeG lesion during synthesis of the repair patch. This leads to a repetitive futile process that likely allows the formation of long stretches of gapped DNA that interferes with DNA replication causing DNA double-strand breaks that in turn trigger apoptosis (for review see ref. 10). In fact, Mgmt-deficient cells in vitro (21,23) and Mgmt-null mice (22,24) are highly sensitive to the toxic effect of S41 methylating agents compared with isogenic Mgmt-expressing cells and individuals. Further support for this model was provided by mismatch repair-deficient cells and mice, which are highly refractory to the killing effect of S41 methylating agents (25). As expected, Mgmt/mismatch repair-double-knockout mice are resistant to the toxic effect of S41 methylating agents, but at the same time show a high tumor incidence upon methylating agent treatment (22,26).

While these studies clearly demonstrated that O6MeG is a key node in cancer formation and Mgmt most important in its defense, the role of N-methylation products in carcinogenesis has not yet been elucidated in detail. N-methylation products such as N7-methylguanine, N3-methyladenine and N3-methylguanine are the major adducts formed in the DNA by both S41 and S42 alkylating agents, amounting to 70, 9 and 2%, respectively, of total methylation products induced in the DNA by MNU in vitro (7). These adducts are repaired by base excision repair (BER) (for review see ref. 27) that represents the major pathway for their removal from DNA (28). No human repair-deficient

Abbreviations: AOM, azoxymethane; BER, base excision repair; DKO, double knockout; DSS, dextran sulfate sodium; Mgmt, O6-methylguanine-DNA methyltransferase; MNU, N-methyl-N-nitrosourea; O6MeG, O6-methylguanine; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling; WT, wild-type.
disorders have been described so far suffering from a complete deficiency in BER, which may be taken to indicate that BER is essential for human development and survival.

The N-methylpurines noted above are recognized and removed from DNA by N-methylpurine-DNA glycosylase (MPG, alias N-alkylpurine-DNA glycosylase, Aag). Aag is a type I DNA glycosylase that, upon release of the methylpurine from the DNA, leaves an abasic site in the DNA that is subsequently repaired by the other components of BER (for review see ref. 29). Aag-null mice are viable and, similar to Mgmt-null mice (18), do not show a spontaneous pathological phenotype (30). Mouse fibroblasts derived from Aag-null mice are sensitive to methylating agents (31) indicating that in this cell type, unrepaired N-methylpurines contribute to the cytotoxicity of methylating agents. Interestingly, Aag-deficient mice treated with methyl methanesulfonate that produces predominantly base N-methylations do not suffer from retinal degeneration, whereas WT mice do (32). This indicates that in some cell types in the body, even in the absence of replication, BER intermediates may cause cytotoxic effects, whereas non-repaired N-methylated bases can be tolerated to some extent.

Although it is clear that N-methylpurines are toxic and genotoxic (33), the contribution to carcinogenicity of N-methylated bases has been a matter of controversy for many years. Thus, S9-type agents producing predominantly N-methylations such as methyl methanesulfonate exhibit only weak carcinogenic potency (34) and were not tumor initiating in two-stage skin carcinogenesis, but rather triggered tumor promotion (35). On the other hand, the finding that Aag-deficient mice are more resistant than WT mice to retinal degeneration following methyl methanesulfonate (32) indicates that organ specificity in the genotoxic and putative carcinogenic response to methylating agents has to be taken into account. Here, we ascertained the response of Aag-null mice to colon cancer formation, and compared it with Mgmt-null mice and Aag/Mgmt-double knockouts (DKO), lacking both DNA repair proteins. We made use of mini-colonoscopy where neoplastic changes in the colon can be detected from very early stages without killing the animals (36). We demonstrate that Aag-deficient mice are more susceptible than Mgmt-deficient mice to colon cancer formation induced by a low non-toxic dose of the S9,1 methylating agent AOM followed by promotion with dextran sulfate sodium (DSS). Our data demonstrate that not only repair of O6MeG by Mgmt but also the repair of N-methylation lesions by Aag is highly important for the defense against colon cancer.

Materials and methods

Mice and induction of colorectal carcinogenesis

Mgmt- and Aag-null mice on a C57BL/6 background were described previously (18,30). Twelve- to fourteen-week-old sex-matched Mgmt, Aag, Mgmt/ Aag-double-null (DKO) and C57BL/6 WT control mice were used in the study. The genotype was checked routinely by PCR. Animal protocols were approved by the Animal Care and Use Committee of the University of Mainz. DSS-induced colitis or colitis-associated colorectal cancer was performed as described previously (37) and outlined in Figure 1A. In brief, mice received a single intraperitoneal injection of the mutagenic agent AOM (Sigma–Aldrich, Deisenhofen, Germany) in phosphate-buffered saline (PBS) (3 or 10 mg/kg body weight; freshly prepared before administration) on day 0. Starting on day 2, colitis was induced by two cycles of 1 % DSS. For analysis of toxic dose and acute inflammation (38), 2 % DSS (MP Biomedicals, Illkirch, France) was administered in drinking water followed by normal drinking water.

Mouse endoscopy

For the continuous monitoring of colonic inflammation and tumorigenesis, a high-resolution video miniendoscope (Karl Storz, Tuttingen, Germany) was used. Endoscopic scoring of colitis activity was based on the murine endoscopic index of colitis severity scoring system that includes classification of mucosal translucency, vascularity, granularity, fibrin deposition and stool consistency (36). In some experiments, the Exera II CV-180 narrow band imaging system from Olympus was used to analyse colonic changes in the microvasculature and changes of the crypt pattern (38). Scoring of tumor development was based on tumor size and the number of tumors, as described previously (36). Briefly, tumor sizes were graded as grade 1 (very small but detectable tumor), grade 2 (tumor covering up to one-eighth of the colonic circumference), grade 3 (tumor covering up to a quarter of the colonic circumference), grade 4 (tumor covering up to half of the colonic circumference) and grade 5 (tumor covering more than half of the colonic circumference).

Histopathology

Colons were removed, flushed with PBS, fixed in 10% neutral buffered formalin overnight, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin for histopathological evaluation of inflammation and neoplasia. The degree of inflammation was graded semi-quantitatively on a scale from 0 to 6 in a blinded fashion as described previously (38). The inflammation score was combined of inflammatory cell infiltration ranging from 0 to 3 and tissue damage ranging from 0 to 3. In some experiments, longitudinally opened colons were stained for 5 min with methylene blue solution (1%) for macroscopic analysis and evaluation of aberrant crypt foci.

Detection of apoptosis

For detection of AOM-induced apoptosis, mice were injected with 10 mg/kg AOM in PBS. Forty-eight hours later, colons were removed, flushed with PBS, fixed in 10% neutral buffered formalin overnight, embedded in paraffin and sectioned at 5 μm thickness. Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay using the fluorescein in situ cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions.

Statistics

Assays were performed as outlined in the legend of the figures.

Results

Initially, mice WT and knockout for Mgmt, Aag and Mgmt/Aag were treated with a single dose of AOM (10 mg/kg), which was insufficient to induce tumors on its own, followed by two cycles with DSS (2% in the drinking water) (for the experimental protocol see Figure 1A). As shown in Figure 1B, nearly all WT mice survived the treatment, whereas the knockout individuals died to different extent. Aag−/− mice were not significantly more susceptible than WT mice showing >70%
survival after 14 days, whereas Mgmtn-/ mice display high mortality showing ~15% survival (Figure 1B). Mgmtn-/ and Mgmtn-/-/Aag--/ (designated as DKO) were not significantly different in their toxic response suggesting that Mgmtn is particularly important for protection against AOM/DSS-induced toxicity. To obtain information about the pathological events leading to high mortality in Mgmt-deficient mice, we analyzed the weight development and mucosal alterations in AOM/DSS-treated animals. As a result, both Mgmtn-/- and DKO mice showed a rapid weight loss that was significantly different from WT and Aag--/ mice, which lost only moderate weight (Figure 2A).

Endoscopic analysis at day 6 of the experiment clearly demonstrated that this severe weight loss after AOM/DSS treatment was associated with strong intestinal damage in Mgmtn-/- and DKO mice (Figure 2B). Most notably, intestinal pathology was characterized by multiple deep ulcerations in Mgmtn-/- and DKO mice (an example is shown in Figure 2C) indicating that severe intestinal damage is essential for wasting disease and lethality in these mice. Overall, the data show that Mgmtn-/- mice are more sensitive than Aag--/ mice, and mice deficient in both Mgmt and Aag exhibit sensitivity similar to Mgmtn-/- mice as to the toxic effect induced by AOM followed by DSS.

Since the use of 2% DSS resulted in 90–100% mortality in the Mgmtn-/- and DKO group in the period between 6 and 10 days after treatment (Fig. 1B), we reduced the dose of the promoter and used 1% DSS, which caused only mild colon inflammation and complete survival in all experimental groups (data not shown). We should note that the first treatment cycle with DSS occurred 2 days after AOM injection in order to avoid any possible interference of the tumor promoter DSS with AOM damage fixation. Animals were weighed twice per week, and at day 60 and 120 following AOM treatment, they were inspected by mini-colonoscopy (36) to determine non-invasively the frequency of neoplastic lesions (adenocarcinomas in situ) in the colon (Figure 1A). Examples of colon inspection by mini-colonoscopy at day 60 are shown in Figure 3. The number of tumors per mice after treatment with AOM alone was between 0 and 0.4 in the DKO group and for DSS alone between 0 and 0.2 in the Mgmtn-/- group. This shows that DSS alone (treatment over two cycles) was ineffective in increasing the spontaneous tumor yield significantly, even in the DKO group. Similarly, a single treatment with AOM alone was not sufficient to induce a significant tumor yield both in the WT and the repair knockout mice strains. Only the combination of AOM plus DSS was effective in colon cancer induction. The tumor yield after treatment with a low and a high dose of AOM of 3 and 10 mg/kg, respectively, followed by 1% DSS is presented in Figure 4A and B, respectively. The low dose AOM treatment followed by 1% DSS did not induce colon carcinomas in Mgmtn-/- mice at higher level than in the WT, whereas Aag--/ mice displayed a significantly higher tumor incidence. The DKO individuals exhibited the highest tumor incidence, which was, however, not significantly different from Aag--/ mice (Figure 4A). For the 10 mg/kg AOM dose Mgmtn-/- mice clearly responded with a tumor yield that was significantly higher than in the WT and similar to Aag--/ and DKO mice (Figure 4B). Obviously, with a tumor yield of four to six tumors per individual the saturation level of tumor incidence was reached.

Another end point we used is tumor size, which was again determined by mini-endoscopy. As shown in Figure 4C and D, the tumor size clearly mirrored the tumor yield shown in Figure 4A and B, respectively. Thus, with 3 mg/kg AOM, the average size of tumors was significantly higher in Aag--/ and DKO mice than in Mgmtn-/- and WT. With the high dose of 10 mg/kg AOM, tumors in Mgmtn-/-, Aag--/ and DKO mice had about the same size, indicating again a saturation effect at the maximum tolerable dose. The tumor score (taking into account number and size of tumors per animal; see Materials and Methods) is given in Figure 4E and F. The data shows the same responses for WT and the DNA repair-defective knockout strains as described above. At the end of the experiment, tumors were inspected and histologically defined as carcinoma in situ, an example of which is shown in Figure 5A. Overall, for all end points determined and at low AOM dose level, Aag--/ mice were more susceptible to colon cancer formation than Mgmtn-/- mice.

**Fig. 2.** Increased acute mucosal inflammation in Mgmtn-/- and Mgmtn-/-/Aag-/- versus Aag-/- and WT mice after administration of AOM/DSS. (A) Weight analysis of WT (n = 8), Mgmtn-/- (n = 6), Aag-/- (n = 8) and Mgmtn-/-/Aag-/- (n = 8) mice treated with AOM (10 mg/kg) and 2% DSS. Weight differences between Mgmtn-/- and DKO versus WT and Aag-/- groups were highly significant at day 5 and 6. Differences of Mgmtn-/- versus DKO and WT versus Aag-/- mice were not significant. (B) Analysis of mucosal inflammation by mini-colonoscopy at day 6 after administration of DSS. Data represent mean ± SEM. Statistical analysis was performed using unpaired, two-tailed Student’s t-test. ***P < 0.001, **P < 0.01, *P < 0.05; n.s. not significant. (C) Endoscopic image of an area with severe ulcerative inflammation (labeled by arrow) in DKO mice.
It is striking that Mgmt<sup>−/−</sup> mice displayed at 3 mg/kg AOM a lower tumor response than Aag<sup>−/−</sup> mice. A reasonable explanation might rest on the finding that O<sup>6</sup>MeG is a powerful apoptotic DNA lesion. Thus, it might be surmised that notably in Mgmt<sup>−/−</sup> mice premalignant tumor cells become eliminated by apoptosis triggered by non-repaired O<sup>6</sup>MeG adducts. This elimination mechanism is probably not operative in the WT and Aag<sup>−/−</sup> mice, which are proficient for the repair of O<sup>6</sup>MeG adducts. To check this hypothesis, we inspected the colon of AOM-treated individuals for apoptotic cells. Indeed, Mgmt<sup>−/−</sup> mice exhibited a clearly higher level of apoptotic cells in the colon crypts than Aag<sup>−/−</sup> mice and the WT, a representative example is shown in Figure 5B. The quantification shown in Figure 5C demonstrates that Mgmt<sup>−/−</sup> and DKO mice exhibit upon AOM treatment a dramatically higher level of apoptotic cells per crypt than the WT and Aag<sup>−/−</sup> mice, which supports the hypothesis noted above.

**Discussion**

This study was aimed at elucidating the role of Mgmt and BER in the defense against colon cancer formation. We applied the AOM-DSS protocol, administering a single dose of the initiator AOM followed by two cycles of treatment with the colon-specific tumor promoter DSS. Colon cancer formation was monitored by mini-colonoscopy (36), which has the advantage that individuals need not be killed for colon inspection and neoplastic changes can be detected at an early stage.

Colon cancer formation was monitored by mini-colonoscopy (36) and neoplastic changes can be detected at an early stage. This study was aimed at elucidating the role of Mgmt and BER in the defense against colon cancer formation. We applied the AOM-DSS protocol, administering a single dose of the initiator AOM followed by two cycles of treatment with the colon-specific tumor promoter DSS. Colon cancer formation was monitored by mini-colonoscopy and neoplastic changes can be detected at an early stage.

Using a high dose of DSS (2% in the drinking water for two treatment cycles together with AOM) Mgmt<sup>−/−</sup> mice responded more sensitively than Aag<sup>−/−</sup> mice as to survival. Background experiments showed that repeated cycles of DSS administered at >2% in the drinking water leads to massive epithelial cell apoptosis and, therefore, very probably to a disruption of the colon epithelial barrier. This leads to infiltration of bacteria into the mucosa causing severe intestinal inflammation that clearly contributes to animal death (37,39).

A low-dose DSS (1%), which was used in our experiments, was not toxic, not carcinogenic and caused only mild inflammation, but nevertheless was able to drive the process of colon cancer formation if applied following AOM. We should note that, as shown in a previous study, at very high concentration (2.5%) and long-term exposure (seven cycles), DSS alone can already be active in inducing colon cancer in mice (40). This was taken to indicate that inflammation provoked DNA damage (e.g. by free radical formation) may cause colon carcinogenesis per se. Under these conditions, Aag may exert protection presumably by repairing oxidative DNA lesions (40). As noted above, in the experiments reported here, the DSS concentration in the drinking water (1%) and short-term exposure (two cycles) did not cause severe inflammation in the colon and was ineffective in increasing the frequency of colon carcinomas above the background. Therefore, under the experimental conditions applied the tumorigenic effects in the repair-deficient mice cannot be attributed to DSS. We infer that the lack of repair of methylation lesions induced by AOM is responsible for colon cancer formation. The mechanism of tumor promotion by low concentration of DSS is not entirely known, but inflammation associated increase in cryptal cell proliferation and angiogenesis (41) might represent critical driving components.

The data reported here also show that at the low AOM dose (3 mg/kg) Aag<sup>−/−</sup> mice had a significantly higher tumor response than Mgmt<sup>−/−</sup> mice, which was indistinguishable from the WT. The Mgmt<sup>−/−</sup>/Aag<sup>−/−</sup> mice exhibited a tumor response, which was similar to the Aag<sup>−/−</sup> mice. In contrast, at the high-dose level of AOM (10 mg/kg), tumors were induced at a similar high frequency in the Mgmt<sup>−/−</sup>, Aag<sup>−/−</sup> and Mgmt<sup>−/−</sup>/Aag<sup>−/−</sup>-double knockout individuals. The finding that Aag-deficient animals are even more sensitive than Mgmt-deficient mice to tumor induction at the low AOM dose level indicates that repair of N-alkylated base lesions is highly important for protecting against methylation-induced colon cancer.

Why were Mgmt<sup>−/−</sup> mice not responding to colon cancer formation at low AOM dose level? Colon inspection and TUNEL staining revealed the induction of apoptotic cells in the colon tissue following treatment. It was striking that in Mgmt-lacking mice and in the DKO, significant more apoptotic cells were found in the crypts than in the WT and Aag lacking individuals. Since O<sup>6</sup>MeG is a powerful apoptotic DNA damage in proliferating cells (42,43) causing death at levels...
of <5000 lesions per cell (44), we posit that the low cancer incidence in Mgmt<sup>−/−</sup> mice at the low AOM dose level is probably due to the elimination of a large proportion of cells harboring the lesion. At a higher dose level, elimination is not anymore perfect and an increasing frequency of cells with a high amount of critical DNA damage escape apoptosis. Under these conditions, elimination of genetically damaged cells and mutation fixation might reach an equilibrium, which might explain why the tumor incidence did not exceed four to six carcinomas per treated mice. We should note that a single dose of 15 mg/kg AOM is toxic even in WT mice indicating that the defense brought about by constitutive expression of Mgmt and Aag is overloaded, causing massive cell death and, as a final consequence, systemic toxicity.

Non-repaired N-methylpurines, such as N3-methyladenine and N3-methylguanine, may interfere with replication giving rise to DNA breaks and chromosomal changes in the proliferating colon epithelium and thus may contribute to tumor initiation in colon cells. Also, these adducts are subject to error-prone translesion synthesis that contributes to mutagenesis (45). Non-repaired N7-methylguanine, which is not a replication-blocking lesion, may also contribute to mutagenesis since spontaneous hydrolysis of the adduct leads to apurinic sites that, if not repaired in time, block replication and generate DNA breaks as well (46). Overall, the data presented here demonstrate for the first time that N-methylpurines contribute to colon cancer formation and stress the importance of the BER system in colon cancer protection. This conclusion supports findings obtained in chronic

Fig. 4. Both AAG and MGMT protect from AOM/DSS induced colon carcinogenesis. Mice received a single does of 3 mg/kg or 10 mg/kg AOM followed by repeated treatment with 1% DSS as outlined in Figure 1A. The number of animals treated are as follows: WT (n = 20), Mgmt<sup>−/−</sup> (n = 11), Aag<sup>−/−</sup> (n = 10), Mgmt<sup>−/−</sup>/Aag<sup>−/−</sup> (n = 10). Tumor numbers and their size were evaluated by mini-endoscopy. The tumor size was graded from 1 to 5 as described in materials and methods. (A and B) Tumor number per animal, (C and D) mean tumor size and (E and F) combined tumor score (sum of all size scores/animal) at day 120. Similar data were obtained by inspecting the animals at day 60, although tumors had a smaller size. Statistical analysis was performed using unpaired, two-tailed Student’s t-test. ***P < 0.001, **P < 0.01, *P < 0.05; n.s. not significant.
inflammatory disease of the colon where upregulation of Aag was found to be accompanied by microsatellite instability (47). This is consistent with in vitro studies demonstrating that transfection-mediated overexpression of Aag causes genomic instability upon methylating agent exposure, which was explained by imbalance in the BER pathway (48). Therefore, either lack or overexpression of Aag may be deleterious, increasing genomic instability that drives the process of cancer formation. Thus, proper expression of BER proteins in the colon appears to be more important than hitherto thought.

Colon cancer is the second most frequent cancer and a number of nutritional and genetic factors are known to be causally involved. Much interest has been drawn to polycyclic aromatic hydrocarbons, food-borne heterocyclic amines and heme iron in red and processed meat (49–51). Our study indicates that carcinogens with methylating properties (together with inflammatory stimuli) might play a very important role in colon cancer. While $S_N1$ agents have been considered to be powerful carcinogens because they target the $O^6$-position of guanine, this study shows that $N$-alkylated bases induced by $S_N1$ agents also bear carcinogenic potential. Furthermore, they indicate that $S_N2$-type agents producing mainly $N$-methylations in the DNA might also bear carcinogenic potency in the colon. Overall, the data illuminate the importance of the BER system that, together with Mgmt, constitutes an effective barrier against colon cancer formation and suggest further studies on BER in colon cancer patients.

**Fig. 5.** Tumor histology and intestinal epithelial cell apoptosis following AOM administration. (A) Haematoxylin/eosin-stained colonic cross sections at day 120. Whereas WT mice show normal gut architecture, DKO mice developed high-grade dysplasia consisting of well to moderately differentiated tubular adenocarcinoma or mucinous carcinoma invading into lamina propria and sometimes also into muscularis propria. (B) Mice received 10 mg/kg AOM. Forty-eight hours later, apoptosis was analyzed in colon cross sections by TUNEL staining. Pictures at the right panel demonstrate a magnification of the area labeled in the low magnification picture for Mgmt$^{-/-}$ and Aag$^{-/-}$ individuals. Green labeled spots demonstrate nuclei of cells undergoing apoptosis. (C) Quantification of apoptotic cells in a crypt. Five mice per group were analyzed. Statistical analysis was performed using unpaired, two-tailed Student’s $t$-test. **$P < 0.01$, *$P < 0.05$; n.s. not significant.
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