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Both base excision repair and $O^6$-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 $O^6$-methylguanine ($O^6$MeG) and methylations of the ring nitrogens of purines. The lesions are repaired by $O^6$-methylguanine-DNA methyltransferase (Mgmt) and by enzymes of the base excision repair (BER) pathway, respectively. Whereas $O^6$MeG 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 Mgmt and Aag, to azoxymethane (AOM)-induced colon carcinogenesis, using non-invasive mini-colonoscopy. We demonstrate that both Mgmt- and Aag-null mice show a higher colon cancer frequency than the wild-type. With a single low dose of AOM (3 mg/kg) Aag-null mice showed an even stronger tumor response than the wild-type. With a single low dose of AOM (3 mg/kg), Aag-null mice showed an even stronger tumor response than the wild-type.

**Introduction**

Methylating agents that are widely distributed in the environment cause a significant contribution to tumor formation in human beings. Human exposure to alkylating carcinogens can result from cigarette smoke, fuel combustion, the presence of heterocyclic amines in the diet and from endogenous nitrosation of amides and amines mediated by enteric bacteria and the reaction of secondary amines with nitrite (1–4). Moreover, methylating agents are also used in cancer chemotherapy, e.g. for the treatment of malignant gliomas (5) and metastatic malignant melanomas (6).

Methylating agents, notably the so-called S$_4$1-type agents, produce a wide spectrum of DNA adducts, including $O^6$-methylguanine ($O^6$MeG), $O^6$-methylthymine, N7-methylguanine, N3-methyladenine and N3-methylguanine (7). $O^6$MeG and $O^6$-methylthymine are instructive lesions causing base mispairing and thus lead to point mutations (8). Since $O^6$-methylthymine is induced by S$_4$1 agents in very low amounts (<0.3% of total DNA methylation products compared with 8% of $O^6$MeG (7)), its contribution to mutagenesis and carcinogenesis is usually neglected. Thus, the current paradigm ascribes $O^6$MeG as the major mutagenic and carcinogenic DNA adduct induced by methylating agents. This is supported by the finding that $O^6$-methylguanine-DNA methyltransferase (Mgmt), which repairs $O^6$MeG 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 $O^6$MeG-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 azoxy-methane (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 chloroethylyating 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).

$O^6$MeG is not only a pre-mutagenic and pre-carcinogenic but also a pre-cytotoxic DNA lesion. Toxicity triggered by $O^6$MeG is dependent on the processing of $O^6$MeG/Cyt mismatches by MutSx-dependent mismatch repair, in which thymine is excised and then reinserted opposite the $O^6$MeG 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 S$_4$1 methylating agents compared with the isogenic Mgmt-expressing cells and individuals. Further support for this model was provided by mismatch repair-deficient cells and mice, which are highly refractory to the killing effect of S$_4$1 methylating agents (25). As expected, Mgmt/mismatch repair-double-knockout mice are resistant to the toxic effect of S$_4$1 methylating agents, but at the same time show a high tumor incidence upon methylating agent treatment (22,26).

While these studies clearly demonstrated that $O^6$MeG 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 S$_4$1 and S$_4$2 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
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, Sqh-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 (DKOs), 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 Sqh methylating agent AOM followed by promotion with dextran sulfate sodium (DSS), lacking both DNA repair proteins. Our data demonstrate that not only repair of O^6^MeG 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, Deisenhof, 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 (2% DSS (n = 14 per group). Statistical analysis of survival was performed using log rank test. ***P < 0.001, **P < 0.01; n.s. not significant.

Histopathology

Colonos 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 semiquantitatively 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 µm with methylene blue solution (1%) for macroscopical 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%
Fig. 2. Increased acute mucosal inflammation in Mgmt$^{-/-}$ and Mgmt$^{-/-}$/Aag$^{-/-}$ versus Aag$^{-/-}$ and WT mice after administration of AOM/DSS. (A) Weight analysis of WT ($n = 8$), Mgmt$^{-/-}$ ($n = 6$), Aag$^{-/-}$ ($n = 8$) and Mgmt$^{-/-}$/Aag$^{-/-}$ ($n = 8$) mice treated with AOM (10 mg/kg) and 2% DSS. Weight differences between Mgmt$^{-/-}$ and DKO versus WT and Aag$^{-/-}$ groups were highly significant at day 5 and 6. Differences of Mgmt$^{-/-}$ 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 pre-malignant 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), by two cycles of treatment with the colon-specific tumor promoter DSS. 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.

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Fig. 3. Representative endoscopic images of the distal colon following treatment with AOM/DSS. Mice received treatment with AOM (10 mg/kg) and 1% DSS as outlined in Figure 1A. (A) Representative images of neoplastic colon mucosa obtained by mini-colonoscopy. (B) Optical contrast enhanced mini-colonoscopy of normal non-neoplastic colonic mucosa and (C) tumors (indicated by arrow) at day 120 after treatment with AOM/DSS.
of <5000 lesions per cell (44), we posit that the low cancer incidence in Mgmt−/− 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−/− (n = 11), Aag−/− (n = 10), Mgmt−/−/Aag−/− (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 SN1 agents have been considered to be powerful carcinogens because they target the O6-position of guanine, this study shows that N-alkylated bases induced by SN1 agents also bear carcinogenic potential. Furthermore, they indicate that SN2-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<sup>−/−</sup>- and Aag<sup>−/−</sup> 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.
Conflict of Interest Statement: None declared.

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