DNA Alkylating Agent Protects Against Spontaneous Hepatocellular Carcinoma Regardless of O⁶-Methylguanine-DNA Methyltransferase Status

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Abstract

Hepatocellular carcinoma is increasingly important in the United States as the incidence rate rose over the last 30 years. C3HeB/FeJ mice serve as a unique model to study hepatocellular carcinoma tumorigenesis because they mimic human hepatocellular carcinoma with delayed onset, male gender bias, approximately 50% incidence, and susceptibility to tumorigenesis is mediated through multiple genetic loci. Because a human O⁶-methylguanine-DNA methyltransferase (hMGMT) transgene reduces spontaneous tumorigenesis in this model, we hypothesized that hMGMT would also protect from methylation-induced hepatocarcinogenesis. To test this hypothesis, wild-type and hMGMT transgenic C3HeB/FeJ male mice were treated with two nonfunctional alkylating agents: diethylnitrosamine (DEN; 0.025 µmol/g body weight) on day 12 of life with evaluation for glucose-6-phosphatase-deficient (G6PD) foci at 16, 24, and 32 weeks or N-methyl-N-nitrosurea (MNU; 25 mg MNU/kg body weight) once monthly for 7 months starting at 3 months of age with evaluation for liver tumors at 12 to 15 months of age. No difference in abundance or size of G6PD foci was measured with DEN treatment. In contrast, it was unexpectedly found that MNU reduces liver tumor prevalence in wild-type and hMGMT transgenic mice despite increased tumor prevalence in other tissues. hMGMT and MNU protections were additive, suggesting that MNU protects through a different mechanism, perhaps through the cytotoxic N⁷-alkylguanine and N³-alkyladenine lesions which have low mutagenic potential compared with O⁶-alkylguanine lesions. Together, these results suggest that targeting the repair of cytotoxic lesions may be a good preventative for patients at high risk of developing hepatocellular carcinoma. Cancer Prev Res; 9(3); 245–52. ©2015 AACR.

Introduction

The incidence of hepatocellular carcinoma in the United States has been increasing since the 1980s and is linked with nonviral etiologies such as obesity and cirrhosis (1–3). Potentially curative therapies, including resection, liver transplant, and radiofrequency ablation, are available only for patients diagnosed in early stages of disease. Surveillance of cirrhotic patients with imaging has improved early detection somewhat, but many patients are still diagnosed with hepatocellular carcinoma in advanced stages when chemotherapy is the only available treatment option. Sorafenib, a tyrosine kinase inhibitor, is the only FDA-approved chemotherapeutic agent for treatment of hepatocellular carcinoma at later stages and provides a median overall survival of only 10.3 months (4). Increased understanding of hepatocellular carcinoma development and identification of targets to prevent progression to hepatocellular carcinoma in high-risk patients would be invaluable in reducing hepatocellular carcinoma incidence and death rates.

The C3HeB/FeJ mouse strain is a nontransgenic inbred mouse model for nonviral hepatocellular carcinoma. This model has a number of features in common with human hepatocellular carcinoma, which makes it a good preclinical model. Human hepatocellular carcinoma has a 2:1 to 4:1 male gender predisposition (5), and spontaneous hepatocellular carcinoma arises in male mice of this strain. Hormones seem to play a role in the gender bias of hepatocellular carcinoma in this mouse model; ovariectomized females injected with androgens display an increased prevalence and castrated males given estrogen injections display a reduced incidence (6–8). In humans, incidence of
hepatocellular carcinoma increases as people achieve middle age, the 40s and 50s (9, 10). Male mice develop hepatocellular carcinoma spontaneously in middle age (9–12 months of age) with a prevalence of approximately 50%. As in humans, tumors in the C3HeB/FeJ strain vary in their size, location within the liver, and number of tumors per animal (multiplicity). Histologically, hepatocellular carcinoma in C3HeB/FeJ shows many of the same features as human hepatocellular carcinoma, including hepatotoxosis, nuclear inclusions, and anisokaryosis, with tumor stage ranging from dysplastic nodules to well-differentiated hepatocellular carcinoma to poorly differentiated hepatocellular carcinoma with vascular invasion (unpublished).

Tumor susceptibility in C3H mice results from differences at multiple genetic loci when compared with mouse strains that are not predisposed to liver tumor development. Drinkwater and colleagues (11) identified the first “hepatocarcinogen susceptibility locus” (Hcs) by crossing C3H/HeJ males with C57BL/6 females and identified a genetic locus that segregates with tumor prevalence following a challenge with diethylnitrosamine (DEN) or ethylnitrosourea (ENU), both of which produce O6-ethylguanine (O6eG) lesions in DNA (12). Hcs locus 7 (Hcs7), is present on chromosome 1 and accounts for approximately 85% of the difference in tumor induction between C3H and C57BL/6 strains after challenge with an ethylating agent (11, 13). A total of 7 Hcs loci have been identified using congenic mice (14, 15). The C3HeB/FeJ strain used in our studies was derived from the C3H/HeJ strain by ovarian transplantation to eliminate endogenous mouse mammary tumor virus (16). Thus, it is reasonable to expect that there are multiple genetic loci that contribute to the development of hepatocellular carcinoma in the C3HeB/FeJ mice. Denlinger and colleagues (17) showed that treatment of C3HeB/FeJ mice with N-methyl-N-nitrosourea (ENU) or ENU induced squamous cell carcinoma of the stomach, pulmonary adenomas, neurogenic tumors, and others. O6-ethylguanine DNA methyltransferase (MGMT) is a stoichiometric-acting DNA repair protein that repairs O6-ethylguanine (O6eGm) lesions, a highly mutagenic DNA lesion induced by monofunctional methylating agents such as MNU (12, 18, 19). We previously established the role of MGMT in hepatocarcinogenesis.

**Materials and Methods**

**Animals**

Production, initial characterization, and genotyping of hMGMT transgenic mice were described previously (22). The hMGMT transgenic mice (LC261 and LC221 lines) used in this study were produced and maintained in the inbred C3HHe/Feto strain. Wild-type C3HHe/Feto males were obtained from Jackson Laboratories or from in-house matings. All animals were maintained in an Association for the Assessment and Accreditation of Laboratory Animal Care–accredited facility and were specific pathogen-free, including *Helicobacter hepaticus*. All manipulations were approved by the Institutional Animal Care and Use Committee prior to implementation. Mice were fed standard laboratory chow *ad libitum* and housed in microisolator-topped cages under 12:12 light-dark cycles.

**Spontaneous hepatocellular carcinoma**

Animals were group-housed in microisolator-topped cages and fed standard mouse chow *ad libitum*. Each was observed daily to detect signs of illness. Animals were humanely euthanized at 15 (Experiment 1) or 12 (Experiment 2) months of age or when moribund, whichever occurred first. All tumors were scored and assessed for size and the number per animal. Sixty-four animals were assigned to the drug-treatment groups in Experiment 2 to insure sufficient animals to accommodate accidental losses so that at least 58 animals survived in each transgenic line to have a power of approximately 0.8 to detect a change from 50% prevalence in control mice and 25% prevalence in hMGMT transgenic animals (two-tailed test, α = 0.05).

**Hepatocyte preparation, culture, and cytotoxicity measurements**

Hepatocytes were isolated by collagenase perfusion as previously described (23). After anesthetization, a longitudinal incision was made and the liver perfused through the hepatic portal vein with calcium-free Earle balanced salt solution (5.4 mmol/L KCl, 116 mmol/L NaCl, 0.8 mmol/L MgSO4, 1.6 mmol/L NaHCO3, 1.0 mmol/L NaH2PO4, 5.5 mmol/L D-glucose, pH 7.4, 37 °C) containing 25 mmol/L Hepes and 0.5 mmol/L EGTA (Sigma Chemical, Co.). Viable cell yield using 0.05% collagenase (Boehringer Mannheim Biochemica) digestion was routinely approximately 7 × 10^7/animal as determined by trypan blue exclusion. Plates and dishes were prepared by pretreating with a collagen solution (0.5 mg/mL, Sigma Chemical, Co.) mixed with Swim-77 medium (Sigma Chemical, Co.), containing penicillin–streptomycin solution (10,000 U/mL penicillin and 10 mg/mL streptomycin; Sigma Chemical, Co.) at a ratio of 1:8.

Freshly isolated hepatocytes were diluted to a concentration of 5 × 10^4 viable cells/100 μL in L-15 medium/ITS (mixed in a ratio of 100:1; Sigma Chemical, Co., and Collaborative Biomedical Products, respectively). One hundred microliters of the cell suspension were dispensed into 96-well tissue culture plates. Hepatocytes were incubated at 37 °C without CO2 for 4 hours.

MNU (Sigma Chemical, Co.; 10 mg/mL) was prepared fresh in solution A (1.4 mol/L NaCl, 54 mmol/L KCl, 55.5 mmol/L Dextrose, 42 mmol/L NaHCO3; 37 °C) each time it was used. All MNU preparations were protected from light. Twenty to 24 hours after plating, medium was removed from the 96-well tissue culture plates and washed gently two times with solution A. Hepatocytes were subsequently treated with 100 μL of solution A containing 0.0, 0.1, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 7.5, and 10 mg/mL MNU. Cells were treated for 60 minutes at 37 °C in a sealed modular incubator chamber. Afterwards, cells were washed three times with solution A, refed with fresh L-15 medium/ITS and incubated at 37 °C. Twenty to twenty-four hours after MNU treatment, cell viability was quantified with an 3-(4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (24) using the cell proliferation kit I (Boehringer Mannheim Biochemica) according to the manufacturer’s protocol. Absorbance was determined at 550 nm with the reference wavelength at 690 nm using a SLT Lab Instrument 400 ATC microplate reader.
Individual data points obtained by the MTT assay represent the mean of a minimum of 8 wells for each MNU treatment. Treatments were performed in duplicate from three independent experiments. The surviving fraction of each group was plotted as a function of MNU concentration.

DEN treatment of mice

This assay was performed as described in ref. 25. Mice were randomly assigned to untreated control or DEN treatment groups. C3HeB/FeJ wild-type and hMGMT transgenic male mice, lines LC26I and LC22I, were injected intraperitoneally with 0.025 μmol/g body weight on day 12. At 16, 24, and 32 weeks of age, mice were sacrificed, livers were excised, and histology sections were prepared from frozen liver tissue. Foci of glucose 6-phosphatase-depleted activity were determined by IHC and compared with hematoxylin and eosin (H&E)-stained sections and quantitated as described previously (25).

MNU treatment of mice

MNU (Sigma Chemical Co.) was prepared fresh for each use in phosphate/citrate buffered saline at pH 4.2 with constant protection from light.

Experiment 1. MNU was delivered by tail vein injections monthly for 7 months starting at 3 months of age. A total of 25 mg MNU/kg body weight was delivered at each injection based on the body weight at the first injection (17). A total of 125 mg MNU/kg body weight or buffer alone was injected into each animal. Mice were humanely euthanized when moribund or at 15 months of age, whichever occurred first.

Experiment 2. Mice were treated identically as in Experiment 1 except that MNU was delivered by intraperitoneal (i.p.) injection, the injection was based on the current body weight, and mice were humanely euthanized when moribund or at 12 months of age, whichever occurred first.

Tissue collection, IHC, and histologic analyses

A board-certified veterinary pathologist performed complete necropsies on all animals in Experiment 1 of MNU treatment. Visible tumors were removed, formalin-fixed, paraffin-embedded, and sectioned; adjacent sections were processed for (i) H&E staining, (ii) IHC with monoclonal anti-hMGMT (1:100; Kamiya Biomedical Company), (iii) polyclonal antiserum against hMGMT and mMgmt (1:200; Novus Biologicals), or (iv) antibody against mouse Ki67 (1:50; Abcam). The pathologist reviewed all H&E slides. Six 20 × fields of view of each Ki67-stained tumor were examined for cells with positive staining. Whenever possible, 300 tumor cells and 300 nearby normal hepatocytes were scored for the presence of brown precipitate in the nucleus to quantify the proportion of hMGMT, hMGMT/mMgmt, or Ki67-positive cells. Slides were evaluated and imaged using Zeiss Axio Imager A1 and Zeiss AxioCam HRC with Zeiss AxioVision 4.5 Sp1 software.

Statistical analyses

Comparisons of tumor prevalence were carried out using the χ² test for independence. Where required, because of low expected frequencies, exact P values were computed using StatXact 3.0.2 (Cytel Corp.) or NCSS 9 (NCSS). Means and SEs were computed using the fraction of immunohistochemical-positive cells. Afterward, the fractions of positive cells were compared among cell lines, treatments, and tissues using analysis of variance for repeated measurements. Comparisons among means were Bonferroni-adjusted. P values < 0.05 were considered significant. Cytotoxicity data were analyzed using ANOVA for randomized complete blocks. Each experiment was considered as a block. Logarithms were analyzed to better satisfy the assumptions underlying the ANOVA. Means and SEs were computed from untransformed data. The MNU concentration of 0 was not included in the ANOVA.

Results

Male mice were treated perinatally with DEN, and areas of glucose 6-phosphatase–deficient (G6PD) foci, preneoplastic liver lesions that correlate with subsequent liver tumors if allowed to continue to grow (25), were identified, counted, and measured for each liver. The number and volume fraction of G6PD foci were similar in the livers of two independent lines of hMGMT (LC26I and LC22I lines) transgenic and wild-type mice (Supplementary Table S1). DEN-treated mice displayed more G6PD foci, an increased volume fraction, number of foci per cubic centimeter of liver, number of foci per liver, and number of mice with foci compared with control mice.

DEN induces 12% N7-ethylguanines, 8% O6eG, 4% N3-ethyladenine lesions, and ethylphosphate lesions, the most common adduct formed (53%; refs. 12, 18) as a proportion of total damage resulting from alkylation of DNA. MNU induces a similar percentage of O6mG lesions; 7.5%) but a much greater percentage of N7 methylguanine (N7mG; 68%) and N3 methyladenine (N3mA; 8%) adducts (12, 18) as proportions of total damage. O6mG lesions have one of the highest mutagenic potentials of the lesions produced by monofunctional methylating agents, whereas N7-alkylguanine and N3-alkyladenine adducts are more likely to be cytotoxic if they go unrepaired (12). O6eG lesions such as those produced by DEN are repaired by hMGMT at approximately 1/10th to 1/150th of the rate as for O6mG lesions produced by MNU (26, 27). Therefore, mice were treated with MNU to analyze the effects of an alkylating agent that produces DNA damage that is efficiently repaired by MGMT, namely, O6mG. Reduced tumor prevalence after MNU treatment was observed for wild type (Fig. 1, from 30% to 12% after MNU treatment) and hMGMT transgenic mice (Fig. 1; 0% in LC22I and 11% in LC26I lines after MNU treatment). An increase in prevalence of other cancers was observed with MNU treatment in wild-type and hMGMT transgenic mice. Stomach cancers were the most prevalent (Fig. 1), followed by intestinal, lung, and then lymphatic cancers (Supplementary Fig. S1).

We previously demonstrated that a large fraction of tumor cells in spontaneous tumors of hMGMT transgenic mice lacks detectable hMGMT expression compared with normal liver of these transgenic mice (20). To test if this also occurs in tumors that develop with MNU treatment, IHC was used to detect MGMT expression in liver tumors versus normal liver from MNU-treated mice. Polyclonal MGMT antibody detected total MGMT expression (mouse and human proteins; ref. 20) whereas the monoclonal antibody directed against hMGMT enabled us to detect expression of hMGMT only. Tumors that arose spontaneously and in MNU-treated mice all displayed a lower proportion of cells staining positive for hMGMT/mMgmt with the polyclonal antibody; however, only tumors arising in MNU-treated LC26I line of hMGMT transgenic mice displayed significantly lower positive
staining compared with matched normal liver (Table 1). Tumors that arose spontaneously in hMGMT transgenic mice displayed an approximately 70% lower proportion of cells positive for hMGMT (Table 1) but this did not reach statistical significance.

Because the reduction in tumor prevalence with MNU treatment in wild-type mice was unexpected, a second experiment was conducted to test reproducibility and to gather more detailed information on the changes in liver tumor size and multiplicity, using wild-type and hMGMT (LC26I line) transgenic mice. Treatment with MNU reduced the hepatocellular carcinoma prevalence in wild-type (from 56.8% spontaneous to 32.4% after MNU; $P = 0.00178$) and hMGMT transgenic (43.8%–24.6%; $P = 0.0614$) mice (Table 2). These results are similar to what was seen with the first experiment, although the tumor prevalence in untreated hMGMT transgenic mice (43.8%) was higher than previously reported (18%–19%; ref. 20), and therefore was not significantly different from wild-type tumor prevalence. Untreated hMGMT transgenic mice displayed significantly lower spontaneous tumor multiplicity compared with wild type (Table 2; hMGMT: 7.1%; wild type: 36.0%; $P = 0.0367$). MNU treatment significantly reduced the multiplicity of tumors in wild-type mice (Table 2; 20.5%–6.8%; $P = 0.01022$). The percentage of hMGMT tumor-bearing mice with multiple tumors was similar to MNU treatment (Table 2), likely because it was already so low in untreated hMGMT mice. Although there was a decrease in tumor prevalence with MNU treatment, there was an increase in the mean and median total tumor volume with MNU treatment in hMGMT transgenic mice (Table 2). In wild-type mice, there was a similar increase in the mean total tumor volume (Table 2); however, this is due to one mouse with a total tumor volume of ten times the average (7704 mm$^3$; Fig. 2). In general, tumor burden was statistically similar across groups (Table 2), although this may be due to the large variability in total tumor volume within the groups (Fig. 2).

One possible explanation for the lower liver tumor prevalence with MNU treatment is that the DNA damage induced by MNU resulted in cell death in preneoplastic cells thereby precluding carcinogenesis. To directly test the effect of MNU treatment on cell viability, primary hepatocytes from hMGMT transgenic and wild-type mice were isolated, treated with MNU in vitro, and cell viability was measured (Fig. 3). Similar sensitivities to MNU treatment were detected and both demonstrated hormesis, a common dual-phases response to exogenous agents with initial stimulation followed by growth inhibition or cell death. Overall, wild type and hMGMT primary hepatocytes displayed a similar dose-dependent reduction of cell viability with increasing doses of MNU.

Because MNU-treated hMGMT transgenic mice displayed greater median total tumor volume despite lower overall prevalence, we hypothesized that cellular proliferation was greater in MNU-treated tumors compared with untreated tumors. To test this, IHC for Ki67 was performed on fixed tumor tissues. Very few cells were positive for Ki67 staining, regardless of whether they were from mice treated with MNU or solvent control, or from hMGMT transgenic or wild-type mice (Fig. 4A). One tumor displayed foci of positive nuclear staining in cells near the periphery of the tumor (Fig. 4B). A few mitotic figures seen within the tumors stained

Table 1. Proportion of Mgmt/mMgmt positive cells in tumors compared with normal liver of mice treated intravenously with MNU (MNU Experiment 1)

<table>
<thead>
<tr>
<th>Treatment group</th>
<th># Tissues analyzed*</th>
<th>% Positive cells for hMGMT/mMgmt</th>
<th>% Positive cells for hMGMT</th>
<th>% Positive cells for hMGMT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Tumor</td>
<td>Normal</td>
<td>Tumor</td>
</tr>
<tr>
<td>C3HeB/C6</td>
<td>6</td>
<td>6</td>
<td>73.9 ± 4.3</td>
<td>50.1 ± 12.9</td>
</tr>
<tr>
<td>+MNU</td>
<td>2</td>
<td>2</td>
<td>77.7 ± 11.6</td>
<td>46.0 ± 36.8</td>
</tr>
<tr>
<td>LC22I</td>
<td>2</td>
<td>3</td>
<td>77.0 ± 9.3</td>
<td>60.9 ± 11.7</td>
</tr>
<tr>
<td>+MNU</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>LC26I</td>
<td>5</td>
<td>5</td>
<td>59.6 ± 7.9</td>
<td>33.2 ± 11.9</td>
</tr>
<tr>
<td>+MNU</td>
<td>4</td>
<td>4</td>
<td>80.6 ± 4.0</td>
<td>28.8f ± 14.2</td>
</tr>
</tbody>
</table>

NOTE: Data are presented as means ± SEM.

Abbreviations: ND, not determined; NA, not available.

*Indicates statistically significant.

bIndicates tumors for which the proportion of Mgmt/MGMT-positive cells was ≤50% of nearby normal tissue.

Figure 1. Prevalence of hepatocellular carcinoma is reduced with MNU treatment, despite an increase in gastric squamous cell carcinoma, in wild-type and hMGMT (LC22I and LC26I lines) mice (MNU Experiment 1). Tumor prevalence was calculated as the percent of mice with one or more hepatocellular carcinoma or gastric squamous cell carcinoma tumors for each genotype and treatment. * , significantly different from C3HeB untreated control group.
Alkylation Damage Protects against Hepatocellular Carcinoma

Table 2. Tumor prevalence is reduced with i.p. MNU treatment in both wild-type and hMGMT (LC26I line) C3HeB/FeJ mice (MNU Experiment 2)

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Total # mice analyzed</th>
<th># mice with tumors</th>
<th>% with tumors</th>
<th># mice with multiple tumors</th>
<th>% of tumor bearing mice with multiple tumors</th>
<th>% of all mice with multiple tumors</th>
<th>Total tumor burden (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3HeB/FeJ</td>
<td>88</td>
<td>50</td>
<td>56.8%</td>
<td>18</td>
<td>36.0%</td>
<td>20.5%</td>
<td>424</td>
</tr>
<tr>
<td>+MNU</td>
<td>74</td>
<td>24</td>
<td>32.4%</td>
<td>5</td>
<td>20.8%</td>
<td>6.8%</td>
<td>726</td>
</tr>
<tr>
<td>LC26I</td>
<td>32</td>
<td>14</td>
<td>43.8%</td>
<td>1</td>
<td>7.1%</td>
<td>3.1%</td>
<td>208</td>
</tr>
<tr>
<td>+MNU</td>
<td>57</td>
<td>14</td>
<td>24.6%</td>
<td>3</td>
<td>21.4%</td>
<td>5.3%</td>
<td>400</td>
</tr>
</tbody>
</table>

*Indicates statistically significant difference of percentage of mice with tumors between C3HeB/FeJ with MNU compared with untreated wild-type mice (P = 0.0078).

A lower tumor prevalence was observed in wild-type and hMGMT transgenic mice treated with MNU. The effects of the transgene and MNU treatment were additive. When the results from Experiment 1 and 2 are combined (Supplementary Table S2), tumor prevalence was reduced by 13% with the hMGMT transgene, which was a marginally significant effect. Furthermore, with MNU, tumor prevalence is reduced an additional 20.9% in hMGMT transgenic mice, similar to the 28.5% reduction seen in wild-type mice with MNU treatment (Supplementary Table S2). This additive effect implies the mechanisms through which the hMGMT transgene and MNU protect against tumorigenesis are independent. The hMGMT transgene is thought to provide protection by increasing the repair of mutagenic O6mG lesions (20).

In support of independent mechanisms, there was a significant loss of hMGMT/mMgmt expression in tumors that arose from hMGMT transgenic mice treated with MNU compared with matched normal liver and a tendency toward loss of expression in the untreated transgenic groups (>70% reduction in hMGMT positive staining cells but did not reach significance likely due to group size). These data support that in the transgenic mice, protection from the hMGMT transgene expression is lost during tumorigenesis. However, a significant loss of mMgmt expression is not observed in the wild-type mice with or without MNU, which suggests that MGMT expression is not a driving factor for protection against tumorigenesis provided by MNU. This conclusion is further supported by the dose-dependent kill curves of primary hepatocytes from wild-type and hMGMT transgenic mice. The hMGMT transgene does not provide protection from the cytotoxicity of MNU, suggesting therefore that death induced by MNU is not due to O6mG lesions in cells.

In contrast to the tumor preventative effect of MNU, DEN caused increased prevalence and volume of preneoplastic G6PD foci in wild-type and transgenic mice, with no protective effect imparted by the hMGMT transgene. It was shown previously that all perinatally DEN-treated C3H mice develop liver tumors if allowed to survive long enough and that G6PD foci will develop into tumors if allowed to progress (11). DEN is administered perinatally, when hepatocytes are proliferative, to induce G6PD foci and eventual tumorigenesis (30). However, the hMGMT transgene may or may not have been expressed at this time because it has been shown previously that genes driven by the transferrin promoter are not expressed in the liver until between 10 and 20 days of age (31). If O’-alkylguanane repair is key for preventing DEN-induced carcinogenesis, the weak or absent transgene expression may explain the lack of difference between DEN-treated wild-type and hMGMT transgenic C3HeB/FeJ mice. In addition, DEN produces O6mG lesions which are repaired less
efficient overall than O6mG lesions by hMGMT (26, 27). Low-efficiency repair of O6eG could also have contributed to the similar outcomes for the wild-type and hMGMT transgenic mice, whether or not hMGMT was expressed at the time of treatment. Unrepaired O6mG lesions can be recognized by mismatch repair and lead to apoptosis (32). On the other hand, the toxicity of O6eG lesions is independent of mismatch repair (33). This could also potentially explain the differences in response to DEN versus MNU.

However, the data suggest that the O6mG lesion and its repair may not be the mechanism for MNU's cancer preventative effect. Although O6mG is one of the most mutagenic lesions induced by monofunctional methylating agents, it makes up a minority of the DNA damage. Other lesions, such as N7-alkylguanine and N3-alkyladenine adducts, occur in a greater proportion but do not have the high mutagenic potential of the O6mG adduct. Instead, they are more cytotoxic if they are not repaired (32). Interestingly, while DEN produces O6eG in the same proportion as MNU produces O6mG, DEN generates a much lower proportion of N7-alkylguanine and N3-alkyladenine adducts compared with MNU (12). The differences in proportion of N7-alkylguanine and N3-alkyladenine may contribute to the differential effect on tumorigenesis by these two monofunctional alkylating agents.

MNU induces the same lesions and at similar proportions to those induced by temozolomide, the first-line therapy for glioblastoma (12, 34). Although MNU or temozolomide would not be used for chemoprevention, these studies do reveal a potential target for chemoprevention. Targeting the repair of N7- and N3-adducts may have a similar preventative effect. Reduced efficiency
of base excision repair should yield cell death from N7- and N3-adducts because these lesions can lead to cytotoxic abasic sites (32). This may reduce the number of preneoplastic lesions, particularly in high-risk patients. Unlike targeting the repair of O6-alkylguanine lesions, this approach does not carry the same risk of increased carcinogenesis because N7- and N3-adducts do not have as much mutagenic potential (32). Because high-risk patients are already identified to undergo surveillance for development of hepatocellular carcinoma, there is an opportunity to treat with a preventative agent to reduce incidence of hepatocellular carcinoma. As hepatocellular carcinoma is refractory to chemotherapy in the majority of patients, effort toward prevention in high-risk patients may be more effective.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


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