Strain Differences in Mice Highlight the Role of DNA Damage in Neoplasia Induced by Low Dietary Folate¹,²

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Abstract
In earlier work, we showed that low dietary folate induced intestinal tumors in BALB/c mice. In this study, our goal was to examine the effect of the same diets on a strain that is more resistant to tumorigenesis (C57Bl/6). We also questioned whether supplementation of the folate-deficient diet (FD) with betaine, an alternate methyl donor, would influence tumor formation. C57Bl/6 mice were fed the same diets [control diet (CD) with 2 mg folate/kg diet and FD with 0.3 mg folate/kg diet] as those in our previous study for 1 y, but they did not develop tumors. We also fed BALB/c mice the FD or FD supplemented with betaine for 1 y, but there was no change in tumor incidence. To determine the relative contributions of DNA damage and altered methylation patterns, we measured intestinal dUTP:dTTP ratios, phosphorylated histone H2AX (p-H2AX) staining, and global DNA methylation in both strains. Only BALB/c mice showed changes due to diet in dUTP:dTTP (from 2.19 ± 0.20 in CD to 2.77 ± 0.18 in FD; P = 0.05) and in p-H2AX staining (from 14.10 ± 3.59% in CD to 22.40 ± 2.65% in FD; P = 0.054). In BALB/c mice only, FD tended to have less (P = 0.06) global DNA methylation than CD. Although the FD increased plasma homocysteine and the betaine-supplemented FD lowered plasma homocysteine, the latter diet did not reduce tumor incidence. We conclude that plasma homocysteine is not likely to be associated with tumorigenesis in our model. However, DNA damage plays a critical role in initiating tumorigenesis when dietary folate is low and methylation changes may also be contributory. J. Nutr. 138: 653–658, 2008.

Introduction
Epidemiologic studies have suggested that low dietary folate is associated with increased risk for colorectal cancer. To demonstrate a direct causative role of folate deficiency in tumor development, we fed low-folate diets to mice for 1 y and showed that dietary folate deficiency alone was sufficient to induce intestinal tumors in these mice (1). The percentage of mice that developed tumors increased if they also had a null allele in Mthfr, an important enzyme of folate metabolism. Insight into the mechanisms by which folate deficiency transforms the cells of the intestine is limited, although there are several theories. Folates are necessary for the conversion of dUMP to dTMP and for the conversion of homocysteine to methionine, a precursor for methylation reactions through S-adenosylmethionine. Consequently, dietary folate deficiency can reduce availability of thymidine for DNA synthesis/repair and the supply of methyl donors for methylation reactions. Disruptions in folate metabolism can therefore result in DNA double-strand breaks, abnormal DNA methylation patterns, and increased levels of homocysteine, all of which have been associated with cancer development (2–4).

In our earlier study, we used BALB/c mice with and without a null allele in the Mthfr gene. The common 677C→T variant of MTHFR in human populations has been proposed as a risk factor for certain cancers, particularly when dietary folate is low (5,6). A single null allele in our mice (Mthfr+/-) reduces the activity of MTHFR and moderately increases plasma homocysteine concentrations. Consequently, the Mthfr+/- mice are a good model for mild MTHFR deficiency in humans (7).

Different mouse strains have varying susceptibilities to cancer (8,9). In particular, BALB/c strains have been shown to be sensitive to carcinogen-induced colorectal tumors, whereas C57Bl/6 strains are more resistant (10). We therefore chose to examine the effects of our folate-deficient diet (FD) on the C57Bl/6 strain by feeding them the same diets for the same length of time as those in our previous study. Because we have recently crossed the Mthfr null allele from the BALB/c onto the C57Bl/6 background (11), we also examined the effects of low folate in combination with MTHFR deficiency on this strain.

Our previous study found that folate deficiency increased the number of DNA double-strand breaks in the normal intestine of the BALB/c mice. We also found altered expression at the mRNA
and protein levels of 2 DNA damage response genes: polo-like kinase 1 (Plk1) and cell division cycle 25c (Cdc25c) (1). We therefore elected to examine the incidence of DNA double-strand breaks and expression of Plk1 and Cdc25c in this study to determine whether the same mechanisms might be operating in the C57Bl/6 background. We also measured the levels of dUTP compared with dTTP to determine whether folate deficiency resulted in imbalances of nucleotide pools that could lead to uracil misincorporation and DNA damage.

Betaine is an alternate methyl donor in the remethylation of homocysteine to methionine (3) through the action of the enzyme betaine homocysteine methyltransferase. In other work, we supplemented Mthfr+/− and Mthfr−/− mice, with betaine and showed that betaine supplementation significantly decreased plasma homocysteine (12). Subsequently, we showed that betaine supplementation increased methionine levels and slightly reduced DNA hypomethylation in mutant mice (13). We therefore questioned whether betaine supplementation might reduce tumorigenesis due to low dietary folate if hyperhomocysteinemia and/or disruption of methylation reactions were critical to the formation of tumors in our mouse model.

Materials and Methods

Mice. Animal experimentation was approved by the Animal Care Committee of the Montreal Children’s Hospital. Male and female Mthfr+/+ and Mthfr−/− mice, generated in earlier work and backcrossed for at least 10 generations onto C57Bl/6 and BALB/c backgrounds (7,11), were housed at the Montreal Children’s Hospital Research Institute animal facility. After weaning, mice were fed amino acid-defined diets (Harlan Teklad) with all the necessary components recommended by AIN (14). The control diet (CD) contained the recommended amount of folic acid for rodents (2 mg/kg diet) (14) and the FD diet contained 0.5 mg/kg diet. These diets have been used and described in our previous reports and the FD was shown to be effective in lowering folate or increasing homocysteine as expected (11,15). The data for the CD and FD BALB/c mice were previously published (1). For this report, BALB/c mice were given the FD or the FD supplemented with betaine (FDB), which was folate deficient (0.3 mg/kg diet) but contained 2.93 g/kg anhydrous betaine, as described in our previous studies (12,13). All diets contained 1% succinylsulfathiazole, an antibiotic, to prevent generation of folate by intestinal bacteria. Mice were fed these diets for 12 to 14 mo until they were killed by suffocation in a CO2 chamber. Body weights were re-

Global DNA methylation. We used TLC to measure the global amount of DNA methylation at CCGG sites, as previously described (17).

dUTP:dTTP ratio. Free deoxyribonucleotides were separated using an HPLC method previously described (18) with slight modifications. Briefly, frozen preneoplastic intestine was ground to powder, treated with 0.6 mol/L trichloroacetic acid, neutralized with trioclylamine, and injected onto an Econosphere C18 column (particle size 5μm, length 250 × 4.6 mm, Waters instrument part no. 70071). Separation of dUTP and dTTP was achieved by isocratic elution with 100% buffer A (0.1 mol/L NH4H2PO4, 0.33 mol/L KCl, 0.25% methanol, pH 5.35) for 12 min followed by a linear gradient to 25% buffer B (0.1 mol/L NH4H2PO4, 0.4 mol/L KCl, 20% methanol, pH 5.0) for 18 min followed by a linear gradient to 80% buffer B for 10 min, then 10 min of 80% buffer B, followed by reequilibration with 100% buffer A for 20 min. The flow rate for the entire run was 0.8 mL/min. A UV detector monitored peaks at wavelengths of 254 nm and 280 nm. Peaks were assigned by coelution with known standards (Sigma-Aldrich).

Statistical methods. Differences in tumor incidence between groups were assessed with Fisher’s exact test. Within a strain, mice fed the CD and FD were compared using independent sample t tests and paired sample t tests were used when tumors and normal tissue within a mouse were compared. We used 2-way ANOVA to evaluate the effects of strain, diet, genotype, and their interactions, followed by Tukey’s post hoc test. SPSS for Windows (release 10.0.1) was used for analyses. Values are means ± SEM. Differences were considered significant at P < 0.05.

Results

Strain differences

Tumor incidence. We examined 39 CD (24 Mthfr +/+ and 15 Mthfr +/−) and 59 FD (32 Mthfr +/+ and 27 Mthfr +/−) C57Bl/6 mice of both Mthfr genotypes for intestinal tumors. None of the C57Bl/6 mice had intestinal adenocarcinomas, adenomas, or polyps, by gross and histological examination, in contrast to what had been observed in the BALB/c strain (1). Tumor incidence differed between the BALB/c and C57Bl/6 strains (P < 0.05).

Plasma homocysteine. Plasma homocysteine concentrations [an indicator of nutritional folate deficiency (19,20)] were measured in 10 samples per diet group in C57Bl/6 and BALB/c strains, 5 samples for each Mthfr genotype (+/+ and +/−). We observed the expected increases in plasma homocysteine due to the FD and to the Mthfr +/− genotype in both the BALB/c and C57Bl/6 strains (Table 1). Strain, diet, and genotype affected the plasma homocysteine concentration (P < 0.01). It is unclear if the strain differences are biologically relevant, particularly because we do not observe consistent strain differences in homocysteine concentrations in related work (our unpublished data).

Global DNA methylation. Based on the increased plasma homocysteine concentrations in mice fed FD, we hypothesized that the remethylation of homocysteine to methionine was impaired in FD mice of both strains. We therefore examined global DNA methylation levels in 6 CD and FD normal intestines (3 Mthfr +/+ and 3 Mthfr +/− per group) of the 2 strains. In the BALB/c strain, global DNA methylation tended to be greater in CD (77.8 ± 1.1%) than in FD (72.4 ± 1.8%; P = 0.06). In the C57Bl/6 strain, global DNA methylation did not differ between mice fed the CD (60.3 ± 1.2%) and FD (60.9 ± 1.0%).

dUTP:dTTP ratios. We examined dUTP:dTTP ratios in the normal intestine of 10 CD and FD BALB/c and C57Bl/6 mice (5 Mthfr +/+ and 5 Mthfr +/− per diet group; Table 1). dUTP:dTTP ratios tended (P = 0.05) to be greater in FD mice than CD.
TABLE 1  Effect of CD, FD, and Mthfr genotype on plasma homocysteine, dUTP:dTTP ratio, and percent p-H2AX foci in BALB/c and C57Bl/6 mice fed the diets for 1 year

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mthfr genotype</th>
<th>Plasma homocysteine μmol/L</th>
<th>dUTP:dTTP</th>
<th>p-H2AX foci %</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>CD +/+</td>
<td>8.55 ± 1.19</td>
<td>2.19 ± 0.20</td>
<td>14.06 ± 3.59</td>
</tr>
<tr>
<td></td>
<td>CD +/−</td>
<td>13.97 ± 3.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FD +/+</td>
<td>18.93 ± 3.58</td>
<td>2.77 ± 0.18</td>
<td>22.43 ± 2.65</td>
</tr>
<tr>
<td></td>
<td>CD +/−</td>
<td>38.87 ± 5.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57Bl/6</td>
<td>CD +/+</td>
<td>4.99 ± 0.14a</td>
<td>1.21 ± 0.08b</td>
<td>8.95 ± 5.62a</td>
</tr>
<tr>
<td></td>
<td>FD +/+</td>
<td>10.64 ± 0.82a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD +/−</td>
<td>9.90 ± 1.18b</td>
<td>1.27 ± 0.15a</td>
<td>11.18 ± 8.97a</td>
</tr>
<tr>
<td></td>
<td>FD +/−</td>
<td>34.56 ± 2.56b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 5–10, except for percent p-H2AX foci in C57Bl/6 mice, where n = 2. Means in a column with superscripts without a common letter differ, P < 0.05.
2 Strain, diet, and genotype, P < 0.01 by ANOVA. For the C57Bl/6 strain, diet/ genotype interaction P < 0.05 by post hoc test. Means in a column with superscripts without a common letter differ.

BALB/c mice. However, the dUTP:dTTP ratio did not differ between C57Bl/6 mice fed the CD or FD. Both CD and FD C57Bl/6 mice had lower dUTP:dTTP ratios than CD and FD BALB/c mice (P < 0.05). dUTP:dTTP ratios did not differ between Mthfr +/+ and Mthfr +/− mice in either the BALB/c or C57Bl/6 strains.

DNA damage. Based on the results of the dUTP:dTTP ratios, we questioned whether the increase in dUTP levels could lead to double-strand breaks and DNA damage. We examined the crypts of 5 Mthfr +/+ CD and FD BALB/c and 2 Mthfr +/− CD and FD C57Bl/6 mice (Table 1). As in our previous study, percent p-H2AX staining tended (P = 0.054) to be greater in FD mice than CD BALB/c mice. In contrast, the percent p-H2AX foci did not differ between C57Bl/6 mice fed the CD or FD. These findings are consistent with the results of dUTP:dTTP ratios for these strains.

DNA damage response genes. We examined the expression of Plk1 and Cdc25c in 3 CD and 3 FD (all Mthfr +/−) C57Bl/6 mice. mRNA levels analyzed by QRT-PCR did not differ in expression between CD and FD normal intestine in C57Bl/6 mice (ratio FD:CD normal intestine: Plk1, 1.02 ± 0.10; Cdc25c, 0.92 ± 0.10). This is in contrast to the differences observed in BALB/c mice in our previous study. Protein levels of Plk1 and Cdc25c, as examined by immunofluorescence, were highly variable; therefore, we concluded that there was no consistent change due to diet (Fig. 1A,B). In addition to the Cdc25c protein levels, we also examined levels of the phosphorylated form of CDC2, an indicator of Cdc25c activity. Increased levels of phosphorylated CDC2 are indicative of inactive CDC25c and cell cycle arrest (21). Our earlier results on phosphorylated CDC2 levels were consistent with the change in expression of Cdc25c (1). Findings for the C57Bl/6 mice in this study showed that p-CDC2 levels (Cdc25c activity) did not differ between CD and FD normal intestine (Fig. 1C). These results are consistent with the absence of diet-induced mRNA expression changes for Cdc25c and Plk1 in this strain.

Betaine supplementation

Tumor incidence. We used the tumor-susceptible strain, BALB/c, and fed 42 mice the FD (24 Mthfr +/+ and 18 Mthfr +/−) and fed 44 mice the FDB (all Mthfr +/−). There was an 8.3% (2 mice with tumors/24) incidence of tumors in FD Mthfr +/+ mice and a 22.2% (4 mice with tumors/18) incidence of tumors in the FD Mthfr +/− mice. There was an 18.2% (8 mice with tumors/44) tumor incidence in FDB mice, which did not differ from the FD Mthfr +/− incidence. In the FDB mice, we observed polyloid-like hyperplasias, adenomas, and adenocarcinomas; these histologic changes are similar to those observed in FD mice (1) (data not shown).

Plasma homocysteine. We measured plasma homocysteine from 7 FD and 7 FDB Mthfr +/− mice. Plasma homocysteine concentrations did not differ between FD BALB/c mice in this study compared with the concentrations in FD BALB/c mice from the original study. However, the plasma homocysteine concentrations in the FDB mice (12.40 ± 1.05 μmol/L) were lower than those in the FD mice (36.90 ± 4.76 μmol/L; P < 0.001) and did not differ from the concentrations in CD BALB/c Mthfr +/− mice (10.64 ± 0.82 μmol/L; Table 1).

Global DNA methylation. We examined global DNA methylation levels in 6 FD and 6 FDB normal intestines (all Mthfr +/−). Global DNA methylation in FDB mice (66.3% ± 1.3%) did not differ from that of FD mice (67.1% ± 1.4%) in normal intestine.

dUTP:dTTP ratios. We examined potential changes in nucleotide pools in the mice supplemented with betaine in 7 FD and 7 FDB normal intestines (all Mthfr +/−). dUTP:dTTP ratios did not differ between FD mice (2.00 ± 0.28) and FDB mice (2.32 ± 0.23).

DNA damage. To further assess any possible effects of betaine supplementation on DNA damage, we quantified p-H2AX foci in 3 Mthfr +/− mice per group fed the FD (25.49 ± 9.77) and FDB (20.22 ± 4.57). The groups did not differ, consistent with the similar dUTP:dTTP ratios in the 2 groups.

DNA damage response genes. We also examined expression of the DNA damage response genes Plk1 and Cdc25c in 6 FD and 6 FDB normal intestines (all Mthfr +/−). Expression of the mRNA level did not differ between the groups for either Plk1 (ratio FDB:FD normal intestine: 0.96 ± 0.01) or for Cdc25c (ratio FDB:FD intestine: 0.92 ± 0.00). In contrast, in 3 pairs of Mthfr +/− normal intestines and tumors, we observed increased expression of both genes in tumors, as observed in the original report for the FD (1). However, due to low sample size, differences were not significant (FDB tumor:FDB normal intestine for Plk1 = 2.55 ± 0.96, P = 0.167; for Cdc25c = 9.63 ± 6.34, P = 0.171).

Discussion

This study confirms our earlier observation that low dietary folate can induce intestinal tumors and highlights an important mechanism in this process. We observed that the C57Bl/6 strain was resistant to tumorigenesis induced by low folate, in contrast to the BALB/c strain. This conclusion is consistent with reports in the literature that indicate C57Bl mice are more resistant to the BALB/c strain. This conclusion is consistent with reports in the literature that indicate C57Bl mice are more resistant to tumorigenesis induced by low folate, in contrast to the BALB/c strain.
FIGURE 1  Effect of diet on PLK-1 (A), CDC25c (B), and p-CDC2 (C) protein levels in normal intestines from 1-y-old C57Bl/6 mice fed CD or FD (400× magnification). (A–C), Immunofluorescent staining using anti-PLK1, anti-CDC25c, or anti-p-CDC2 antibodies (2nd column), respectively. None of the 3 proteins showed a consistent change in staining intensity between CD and FD intestines. Propidium iodide was used as a nuclear counter-stain (first column). The final column represents negative controls containing secondary antibody, but no primary antibody, for each sample. n = 2 samples per group.
tions in the intestine, we cannot exclude the possibility that the C57Bl/6 mice were resistant to localized folate deficiency in intestinal tissue. Betaine successfully lowered plasma homocysteine in BALB/c mice but did not affect tumor incidence, leading us to conclude that increased plasma homocysteine concentrations are not likely to be associated with tumorigenesis in this model, although we cannot exclude tissue-specific homocysteine differences.

In our initial report, we hypothesized that the FD might lead to uracil misincorporation and DNA double-strand breaks. The constant pressure put on the DNA damage response pathway due to the long-term folate-deficient state could potentially cause an error in the complex regulation of G2M checkpoint control, where PLK1 and CDC25c function (1). In this study, we obtained more direct evidence for increased DNA damage from the increased dUTP:dTTP ratios in the normal intestine of FD BALB/c mice. This observation is in concordance with the increased DNA damage in the normal intestine of the FD BALB/c mice. As previously reported, the expression of Plk1 and Cdc25c in tumors increased compared with normal intestine, suggesting a deregulation of these important cell cycle control genes. In contrast, we did not observe altered nucleotide ratios or an increase in DNA damage in the normal intestines of the FD C57Bl/6 mice. Gene expression changes for Plk1 and Cdc25c were also not evident in normal intestine in this strain. In further support of our argument that DNA damage is critical to folate-related tumorigenesis, the FD and FDB BALB/c mice had similar tumor incidence, dUTP:dTTP ratios, DNA damage, and Plk1 and Cdc25c expression levels in the normal intestine. We observed overexpression of Plk1 and Cdc25c in tumors compared with normal intestine in both dietary groups. These results are consistent with the proposed mechanism of DNA damage-induced transformation due to deregulation of damage response genes. The findings also indicate that betaine does not affect DNA damage levels or tumor incidence in this model.

Based on the increased plasma homocysteine concentrations in FD mice of both strains, we might expect changes in methionine and S-adenosylmethionine concentrations and, therefore, in DNA methylation. In the BALB/c strain, global DNA methylation tended to decrease in the FD compared with CD normal intestines. However, there were no changes in DNA methylation due to diet in the C57Bl/6 strain. Our data are consistent with the hypothesis that DNA damage may occur as an initiating event in tumorigenesis, with altered DNA methylation patterns being a secondary event (22). On the other hand, we cannot exclude the possibility that this strain is also resistant to DNA methylation changes through an unidentified mechanism that is independent of DNA damage. Further study is warranted, particularly pertaining to the methylation state of individual genes (i.e. tumor suppressor and oncogenes), which may be contributing to tumorigenesis in our model.

Because betaine is an alternate methyl donor for remethylation of homocysteine to methionine, betaine supplementation might be associated with increased global DNA methylation (13). However, we did not see any differences in global DNA methylation between the FD and FDB intestines. Because betaine homocysteine methyltransferase, the enzyme that catalyzes betaine-dependent homocysteine remethylation, is expressed primarily in liver and kidney, it is possible that betaine has some local effects in those tissues but no effect in intestine. In a previous study, DNA methylation increased in the brain of Mthfr-deficient mice supplemented with betaine; however, because those mice had sufficient dietary folate, it is difficult to compare these studies (13).

In conclusion, we have created a reproducible model for studying dietary influences on sporadic colorectal cancer. Our data suggest that the mechanism behind low folate-induced tumorigenesis involves an imbalance in nucleotide pools, an increase in DNA damage, and a disruption of G2M checkpoint control. We hypothesize that there is a tumor-promoting environment in the normal intestine with DNA damage and altered methylation patterns in BALB/c mice. In particular, DNA damage appears to be a crucial step in promoting tumor growth as evidenced by the inability of the FD to increase DNA damage in the C57Bl/6 strain and the subsequent lack of tumor formation in that strain. This study highlights how innate genetic variation, such as that between the BALB/c and C57Bl/6 strains, can affect risk for spontaneous tumorigenesis and can serve to elucidate the mechanisms that contribute to the process.

**Literature Cited**


