Dietary folate deficiency suppresses N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats

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Introduction

Folate, a water-soluble B-vitamin and important co-factor in one-carbon metabolism, has recently been identified as an important nutritional factor that may modulate carcinogenesis (1–3). The role of folate in carcinogenesis has been best studied for colorectal cancer (1–3). The majority of over 25 published epidemiological studies indicate that dietary folate intake and blood folate levels are inversely associated with colorectal cancer risk (1–3). Although animal studies are generally supportive of a causal relationship between folate depletion and colorectal cancer risk, these studies have shown that the dose and timing of folate intervention are critical in providing safe and effective chemoprevention; exceptionally high supplemental folate levels (4–6) and folate intervention after microscopic neoplastic foci are established in the colorectal mucosa (7,8) promote, rather than suppress, colorectal carcinogenesis. An accumulating body of evidence suggests that folate status may also play a modulatory role in the development of several other malignancies (e.g. lung, pancreas, stomach, cervix, esophagus, brain and leukemia) (1–3). The precise nature and magnitude of the relationship between folate status and the risk of these malignancies, however, are less clearly defined compared with colorectal cancer.

The relationship between folate status and breast cancer risk has just begun to be reported in the epidemiological literature. Among nine published case-control studies that investigated the relationship between dietary folate intake and breast cancer risk, seven showed either a significant or equivocal inverse relationship that was not statistically significant, that became non-significant after adjustment, or that could not be distinguished from other factors in their relation to risk (9–15), whereas one showed a significant inverse association (16). In some studies, the observed inverse association was further modified by the intake of alcohol and other folate co-factors (e.g. methionine, vitamins B₆ and B₁₂) (12,14,15). One nested case-control study, using stored serum samples, found no association between serum folate and breast cancer risk (18). Two large prospective studies have shown a weak inverse association between the total or dietary intake of folate and breast cancer risk (19,20). These prospective studies, however, have indicated that low intakes of folate increase, whereas high intakes of folate decrease, breast cancer risk among women who regularly consume alcohol (19,20), supporting folate±alcohol interactions in breast carcinogenesis observed in case-control studies (12,14,15). Recently, molecular epidemiologic studies have shown that the C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene may modulate breast cancer risk and that the direction and magnitude of the risk modification are influenced by folate status and alcohol consumption (21–23). MTHFR is a critical enzyme in folate metabolism that catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, thereby playing an important role in DNA

Abbreviations: FPGS, folylpolyglutamate synthetase; MTHFR, methylenetetrahydrofolate reductase; MNU, N-methyl-N-nitrosourea.

Epidemiologic studies have suggested that dietary folate intake is inversely related to breast cancer risk. However, epidemiologic evidence has not been consistent nor has it provided unequivocal support for this purported inverse relationship. This study investigated the effect of dietary folate on N-methyl-N-nitrosourea (MNU)-induced mammary tumorigenesis in rats. Weanling, female Sprague–Dawley rats were fed diets containing either 0 (deficient; n = 22), 2 (basal dietary requirement, control; n = 20) or 8 mg (supplemented; n = 20) folate/kg diet for 30 weeks. At 50 days of age, rats received an i.p. injection of MNU (50 mg/kg body wt). At necropsy, all macroscopic mammary tumors were identified and examined microscopically. The effect of dietary folate on genomic DNA methylation in mammary tumorigenesis was determined by the in vitro methyl acceptance assay. The incidence of mammary adenoma and adenocarcinoma in the folate-deficient group was lower than that of the control and folate-supplemented groups (55 versus 90 and 75%, respectively, P = 0.043). Kaplan–Meier analyses also demonstrated a similar trend in the rates of appearance of either adenoma or adenocarcinoma (P = 0.06). In contrast, folate supplementation did not significantly modulate mammary tumorigenesis compared with the control group. Although mammary tumors were significantly hypomethylated compared with non-neoplastic mammary tissues in each dietary group (P < 0.03), folate status did not significantly affect the extent of DNA methylation. The data suggest that dietary folate deficiency of a moderate degree suppresses, whereas folate supplementation at four times the basal dietary requirement does not significantly modulate, mammary tumorigenesis in this model. The role of folate in mammary tumorigenesis needs to be clarified for safe and effective prevention of breast cancer.
synthesis, maintenance of nucleotide pool balance and DNA methylation (1). The MTHFR C677T polymorphism causes thermolability and reduced MTHFR activity, leading to lower levels of 5-methyltetrahydrofolate, an accumulation of 5,10-methylenetetrahydrofolate, increased plasma homocysteine levels (a sensitive inverse indicator of folate status), changes in cellular composition of one-carbon folate derivatives, and DNA hypomethylation (1).

Two animal studies published to date have suggested that folate may modulate mammary tumorigenesis. In mice with confirmed spontaneous mammary cancer, daily i.v. injections of fermentation Lactobacillus casei factor (pteroylglutamate) significantly regressed pre-existing mammary tumors and decreased new mammary tumor formation and lung metastases (24). Another study employing the N-methyl-N-nitrosourea (MNU) rat model showed that a folate-deficient diet provided during the initiation phase of mammary tumorigenesis significantly reduced tumor multiplicity and increased tumor latency compared with a control and folate-supplemented diet (25). The incidence of mammary tumors, however, was not significantly different among these groups (25). However, several inherent limitations associated with these animal studies including the use of non-standard dietary means to modulate folate status, possible growth retardation of animals, the concomitant use of antibiotics that may independently affect folate levels, and the use of animals that are resistant to chemically induced mammary tumorigenesis preclude a definitive conclusion concerning the effect of folate on mammary tumorigenesis.

Because only few modifiable risk factors for breast cancer exist, recent epidemiological observations which suggest that folate deficiency increases, whereas supplementation reduces, breast cancer risk merit further consideration. Folate is an ideal agent for potential chemoprevention of breast cancer. It is a natural vitamin, inexpensive, virtually free of side effects (26) and possesses biologically plausible mechanisms for cancer prevention (1–3). However, the results from published epidemiological and animal studies have been neither consistent nor convincing. Furthermore, a growing body of evidence suggests that folate possesses the dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention (4–8, 27, 28). Folate deficiency has an inhibitory, whereas supplementation has a promoting, effect on progression of established neoplasms (4–8, 27, 28). In contrast, folate deficiency in normal epithelial tissues appears to predispose them to neoplastic transformation, and modest levels of folate supplementation suppress the development of tumors in normal tissues (4–8, 27, 28). Therefore, the potential effect of folate chemoprevention needs to be clearly elucidated in appropriate animal models before folate supplementation can be considered in humans. Given these considerations, this study investigated the effects of dietary folate deficiency and supplementation on the development and progression of mammary tumors in the well-established MNU rat model of breast cancer. Given the role of folate in DNA methylation, an important epigenetic determinant in carcinogenesis (29, 30), we also investigated whether dietary folate modulates genomic DNA methylation in MNU-induced mammary tumorigenesis. Folate, in the form of 5-methyltetrahydrofolate, is involved in remethylation of homocysteine to methionine, which is a precursor of S-adenosylmethionine (SAM), the primary methyl group donor for most biological methylation reactions (1–3).

Materials and methods

Animals and dietary intervention

This study was approved by the Animal Care Committee of the University of Toronto. Pathogen-free, weaning female Sprague–Dawley rats (~50 g; Charles River Laboratories, St Constant, Quebec, Canada) were randomly assigned to receive an amino acid-defined diet (Dyets, Bethlehem, PA) (31) containing either 0 (n = 22), 2 (n = 20) or 8 (n = 20) mg folic acid/kg diet from weaning at 3 weeks of age for 27 weeks through the MNU treatment (at 50 days of age). Rats were singly housed and maintained at 24 ± 2 °C at 50% humidity with a 12 h light/dark cycle. These diets constitute a standard method of inducing folate deficiency or providing supplemental dietary folate in rodents (31) and have been utilized extensively in previous studies of folate and colorectal cancer (4, 7, 8, 27). The diet containing 0 mg folic acid/kg produces progressive folate deficiency of a moderate degree without anemia, growth retardation or premature death through weeks 3–5, after which systemic folate indicators stabilize (27). Although this diet is completely devoid of folate, severe folate deficiency is not induced because of de novo synthesis of folate by intestinal bacteria, some of which is incorporated into the tissue folate of the host (32). This folate-deficient diet is identical to that associated with an increased risk of colorectal neoplasms in previous animal studies using a chemical colorectal carcinogen or genetically engineered murine models of colorectal cancer (4, 7, 8, 27). Two milligram folic acid per kilogram diet is generally accepted as the basal dietary requirement for rodents (33). The diet containing 8 mg folic acid/kg represents folate supplementation four times the basal dietary requirement. This level of folate was chosen because the 8 mg/kg level has consistently provided a degree of chemoprevention against colorectal cancer in previous rodent studies (4, 7, 27). These diets contained 50 g cellulose/kg, 60% of the carbohydrates, 23% fat (or 10% body weight), and 17% l-amino acids (31). The amount of methyl donors, methionine, choline and vitamin B12, 8.2 g, 2.0 g and 50 μg/kg diet, respectively. The detailed composition of the diets has been published previously (8, 31). Diets and water were provided ad libitum.

MNU administration

Notwithstanding the limitations associated with animal models, the MNU rat model is widely used to determine the effects of dietary factors on mammary tumorigenesis for the following reasons: (i) histological similarities of adenocarcinoma to human breast cancer; (ii) local invasiveness and metastatic potential; (iii) a clear operational distinction between the initiation and promotion stages; and (iv) hormonally dependent mammary tumorigenesis (34–38). At 50 days of age, all rats received one i.p. injection of MNU (50 mg/kg body wt; Sigma Chemical, St Louis, MO). A single injection of MNU/kg has become the standard dosage due to its rapid induction and high incidence of mammary tumors combined with minimal toxicity and a short latency period of 3–6 months (34, 35).

Observation parameters

Body weights were recorded weekly. The daily food consumption of each group was measured on a predetermined day of each week. All rats were palpated for mammary tumors once a week beginning 4 weeks after MNU administration. The number, size and location of each tumor were recorded in a systematic manner that, after histological diagnosis, the time of appearance of the cancers could be determined. All the rats were monitored daily for clinical evidence of illness or morbidity and those approaching a moribund state were promptly killed. In addition, rats with tumor burden exceeding 10% of body weight, tumors >15–20 mm in diameter, tumors that impaired normal movement of the animals and ulcerating tumors were immediately killed during the study.

Sample collection and analysis of mammary tumors

Blood was withdrawn from the lateral tail vein of each rat within a week of MNU injection and from the heart at necropsy and centrifuged at 5000 r.p.m. for 10 min at 4 °C. Serum was stored at −70 °C in 0.5% ascorbic acid for serum folate assay. Given the latency period of 3–6 months associated with a single i.p. MNU injection and the average duration for the systemic and tissue folate levels to stabilize, the rats were killed by carbon dioxide inhalation followed by cervical dislocation at 23 weeks after MNU injection (27 weeks after dietary intervention or 30 weeks of age). The liver from each rat was harvested, snap-frozen and stored at −70 °C for determination of hepatic folate concentration. All macroscopic mammary tumors were counted, excised and weighed, and the diameter of each tumor was measured using a digital caliper for final tumor volume computation in a blinded fashion. One-half of each macroscopic tumor was snap-frozen in liquid nitrogen and stored at −70 °C for DNA extraction. The other half of the tumor was fixed in 10% neutral-buffered formalin, processed in a standard manner for hematoxylin–eosin (H&E) staining and histologically analyzed according to Russo et al. (37) by three experienced pathologists (R.R., C.M. and A.M.) blinded to the study group independently.
In the case of a discrepancy, two similar interpretations were utilized for the final analysis. Normal mammary tissue was also excised at necropsy from each rat, snap-frozen in liquid nitrogen and stored at −70°C for DNA extraction and mammmary tissue folate determination.

**Determination of folate concentration**

Serum folate concentrations were determined by a standard microbiological microtiter plate assay using L.casei (39). Hepatic and normal mammary tissue folate concentrations were measured by the same microbiologic assay (39), utilizing a previously described method for the determination of tissue folates (40).

**DNA extraction**

DNA from normal mammary tissue and mammary tumors was extracted by standard technique using a lysing buffer containing protease K followed by phenol, chloroform and isomyl alcohol organic extraction (41). The size of DNA estimated by agarose-gel electrophoresis was >20 kb in all instances. No RNA contamination was detected on agarose-gel electrophoresis. The final preparations had a ratio of A_{260} to A_{280} between 1.8 and 2.0. The concentration of each DNA sample was determined as the mean of three independent spectrophotometric readings.

**Genomic DNA methylation determination**

The methylation status of cytosine-guanine (CpG) sites in genomic DNA from normal mammary tissue and mammary tumors was determined by the *in vitro* methyl acceptance capacity of DNA using 3H-methyl-SAM as a methyl donor and a prokaryotic CpG DNA methyltransferase, Sss1, as described previously (42,43). The manner in which this assay is performed produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous 3H-methyl incorporation. Briefly, mammary tumor and non-neoplastic mammary gland DNA (500 ng) was incubated with 2.0 μCi of 3H-methyl-SAM (New England Nuclear, Boston, MA), 3 U Sss1 methylase (New England Biolabs, Beverly, MA), and 1× Sss1 methylation buffer [120 mM NaCl, 10 mM Tris–HCl (pH 7.9), 10 mM EDTA, 1 mM dithiothreitol] in a total volume of 30 μl for 1 h at 30°C. The Sss1 was inactivated by incubating at 65°C for 10 min. The *in vitro* methylated DNA was isolated from a 15 μl aliquot of the reaction mixture by filtration on a Whatman DE-81 ion-exchange filter (Fisher Scientific, Springfield, NJ). The DNA was washed three times with 0.5 M sodium phosphate buffer (pH 7.0), air-dried and the radioactivity of the DNA retained in the filters was measured by scintillation counting using a non-aqueous scintillation fluor. The amount of radiolabel bound to a filter from an aliquot of the reaction mixture by filtration on a Whatman DE-81 ion-exchange filter (Fisher Scientific, Springfield, NJ). The DNA was washed three times with 0.5 M sodium phosphate buffer (pH 7.0), air-dried and the radioactivity of the DNA retained in the filters was measured by scintillation counting using a non-aqueous scintillation fluor. The amount of radiolabel bound to a filter from the control and folate-supplemented groups (P < 0.001; Table I) and these levels were comparable with those observed in rats placed on the corresponding diets for 20–24 weeks in previous studies (4,7,27,45). These observations indicate that a sufficient degree of systemic folate deficiency and supplementation was achieved in the folate-deficient and supplemented rats, respectively, at the time of MNU injection and throughout the study period for the determination of the effect of folate status on MNU-induced mammary tumorigenesis. At necropsy, the hepatic folate concentrations of the three dietary groups were significantly different (P < 0.001; Table I), and these levels were comparable with those observed in rats placed on the corresponding diets for 24 weeks in previous studies (27,46). At necropsy, the mean mammary gland folate concentration of the folate-deficient group was significantly lower than the control and folate-supplemented groups (P < 0.001) while no significant difference was observed between the control and folate-supplemented groups (Table I). This observation suggests that mammary gland folate concentrations reached a plateau beyond the 2 mg folate/kg diet. This finding is probably due to the fact that folate accumulation in tissues is limited by the level of folypolyglutamate synthetase (FPGS) activity in the setting of substrate excess (47,48).

**Effects of dietary folate on MNU-induced mammary tumorigenesis**

No rats died prematurely or were killed before necropsy in the three dietary groups for reasons other than the presence of large and/or ulcerating tumors as defined in the Materials and methods section. The prevalence of killed rats was similar among the three groups. Consistent with previous observations made in the MNU-Sprague–Dawley rat model of mammary tumorigenesis (34–38), >90% of macroscopic mammary tumors in the present study were identified histologically as either adenomas (15%) or adenocarcinomas (85%). There was an excellent agreement in histological diagnosis of either adenoma or adenocarcinoma among the three study pathologists (kappa statistic = 0.95). The analyses pertaining to mammary tumors were performed for the combination of the three groups. This finding indicates that folate deficiency in the rats fed 0 mg folate/kg diet was moderate; otherwise, growth retardation or premature death would have occurred (44). The mean daily food consumption, which was determined on a pre-assigned day of each week, was also similar among the three groups.

**Serum, liver and normal mammary gland folate concentrations**

At the time of MNU injection (4 weeks after the start of dietary intervention) and at necropsy (27 weeks after the start of dietary intervention), the mean serum folate concentrations were significantly different among the three groups (P < 0.001; Table I). The mean serum folate concentrations of the three dietary groups at these two time points were comparable with those observed in rats and mice placed on the corresponding diets for 20–24 weeks in previous studies (4,7,27,45). These observations indicate that a sufficient degree of systemic folate deficiency and supplementation was achieved in the folate-deficient and supplemented rats, respectively, at the time of MNU injection and throughout the study period for the determination of the effect of folate status on MNU-induced mammary tumorigenesis. At necropsy, the hepatic folate concentrations of the three dietary groups were significantly different (P < 0.001; Table I), and these levels were comparable with those observed in rats placed on the corresponding diets for 24 weeks in previous studies (27,46). At necropsy, the mean mammary gland folate concentration of the folate-deficient group was significantly lower than the control and folate-supplemented groups (P < 0.001) while no significant difference was observed between the control and folate-supplemented groups (Table I). This observation suggests that mammary gland folate concentrations reached a plateau beyond the 2 mg folate/kg diet. This finding is probably due to the fact that folate accumulation in tissues is limited by the level of folypolyglutamate synthetase (FPGS) activity in the setting of substrate excess (47,48).

**Table I. Serum, hepatic and mammary gland folate concentrations**

<table>
<thead>
<tr>
<th>Diet (mg folate/kg diet)</th>
<th>At the time of MNU injection (4 weeks of dietary intervention)</th>
<th>At necropsy (27 weeks of dietary intervention)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Serum folate (ng/ml)</td>
<td>13.75 ± 1.04*a</td>
<td>68.82 ± 2.65*b</td>
</tr>
<tr>
<td></td>
<td>100.78 ± 2.82*c</td>
<td></td>
</tr>
<tr>
<td>Hepatic folate (μg/g tissue)</td>
<td>10.53 ± 1.16*</td>
<td>49.24 ± 3.16*b</td>
</tr>
<tr>
<td></td>
<td>2.18 ± 0.32*</td>
<td>6.42 ± 0.35*b</td>
</tr>
<tr>
<td>Mammary folate (ng/g tissue)</td>
<td>56.20 ± 7.54*</td>
<td>178.13 ± 29.06*b</td>
</tr>
<tr>
<td></td>
<td>175.94 ± 24.32*b</td>
<td></td>
</tr>
</tbody>
</table>

*aResults are expressed as mean ± SEM. Means in a row with different letters at each time point significantly differ at P < 0.001.
Effects of dietary folate on the incidence, latency, multiplicity, volume and weight of mammary adenocarcinomas and adenomas

Table II. Effects of dietary folate on the incidence, latency, multiplicity, volume and weight of mammary adenocarcinomas and adenomas

<table>
<thead>
<tr>
<th>Diet (mg folate/kg diet)</th>
<th>0</th>
<th>2</th>
<th>8</th>
<th>P-value, ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence (%)</td>
<td>0.57a</td>
<td>0.90b</td>
<td>0.75a,b</td>
<td>0.057</td>
</tr>
<tr>
<td>Incidence (%)</td>
<td>0.55a</td>
<td>0.90b</td>
<td>0.75a,b</td>
<td>0.043</td>
</tr>
<tr>
<td>Mean latency (weeks post-MNU injection)</td>
<td>17.83 ± 1.35</td>
<td>17.06 ± 1.11</td>
<td>15.00 ± 1.36</td>
<td>0.29</td>
</tr>
<tr>
<td>Mean multiplicity</td>
<td>3.67 ± 1.03</td>
<td>2.87 ± 0.53</td>
<td>2.20 ± 0.34</td>
<td>0.72</td>
</tr>
<tr>
<td>Mean volume (cm³)</td>
<td>2.83 ± 0.80</td>
<td>2.88 ± 0.58</td>
<td>1.49 ± 0.39</td>
<td>0.28</td>
</tr>
<tr>
<td>Mean weight (g)</td>
<td>0.86 ± 0.23</td>
<td>1.03 ± 0.23</td>
<td>0.58 ± 0.14</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM. Means in a row with different letters significantly differ at P < 0.02 by between-group comparisons.

Excluding one outlier in the 0 mg folate group, which harbored a total of nine adenocarcinomas and adenomas, strengthened the overall comparison of the incidence, latency, multiplicity, volume and weight of adenocarcinomas and adenomas among the three dietary groups. In contrast, no significant difference in mean latency, multiplicity, volume and weight of adenocarcinomas and adenomas was observed among the three groups whether or not the outlier was included or excluded in the analyses.
mammary adenocarcinomas, which is inversely related to the latency, multiplicity, volume or weight among the three groups. Genomic DNA methylation status
determined by the Wilcoxon signed ranks test at different letters within each dietary group denote significant differences by significant difference in the final incidence and mean tumor latency, multiplicity, volume or weight among the three groups was observed (Table III).

**Genomic DNA methylation status**
As shown in Figure 2, the degree of $[^3]$H-methyl incorporation into DNA of the mammary adenocarcinoma and into DNA from the pair-matched non-neoplastic mammary tissue was not significantly different among the three dietary groups. However, the degree of $[^3]$H-methyl incorporation into DNA of the mammary adenocarcinomas, which is inversely related to the extent of genomic DNA methylation, was 4–5-fold higher than that of non-neoplastic mammary tissue within each dietary group ($P < 0.03$; Figure 2), indicating a significantly lower degree of genomic DNA methylation in adenocarcinomas compared with normal mammary tissue.

**Discussion**
Our data suggest that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats. In contrast, dietary folate supplementation at four times the basal dietary requirement does not appear to modulate mammary tumorigenesis in this model. These observations contradict the generally accepted notion based on epidemiologic evidence, which suggests that folate deficiency enhances, whereas folate supplementation suppresses, the development of breast cancer (9–15,19,20). Epidemiologic evidence available thus far has not been consistent nor has it provided unequivocal support for the purported inverse relationship between folate status and breast cancer risk (9–20). However, none of the published epidemiologic studies has demonstrated a positive association between folate status and breast cancer risk. Some epidemiological studies have suggested that folate status alone may not be sufficient in modifying breast cancer risk. However, with alcohol consumption folate deficiency potentiates, whereas folate supplementation reduces, the risk of breast cancer (12,14,15,19,20). Furthermore, some studies have suggested that folate status may modify breast cancer risk in conjunction with other dietary factors involved in one-carbon metabolism such as methionine, vitamins B$_6$ and B$_{12}$ (15,19). Also, there is evidence that the direction and magnitude of the breast cancer risk modification associated with folate status may depend on the MTHFR C677T polymorphism (21–23).

Our data differ from the promoting and protective effect of folate deficiency and supplementation, respectively, on intestinal tumorigenesis observed in the chemical carcinogen (dimethylhydrazine) and genetically engineered rodent models utilizing the same diets employed in the present study (4,7,27,45). However, some animal studies have suggested that folate status may have the opposite effect on intestinal tumorigenesis depending on the timing and dose of folate intervention (4–8). The contradicting effect of dietary folate on mammary and intestinal tumorigenesis in animal models using the same diets suggests that folate may modulate carcinogenesis in a tissue- and/or carcinogen-specific manner. The results from the present study are, however, consistent with those of a previous study that investigated the effect of dietary folate deficiency and supplementation on initiation and early promotion of MNU-induced mammary tumorigenesis in Fischer 344 rats (25). Baggott and colleagues performed a study in which rats were fed a casein-based AIN-76A diet containing either 0, 2 or 40 mg folic acid/kg diet, or 20 mg folinic acid/kg diet at weaning (27 days of age) for 30 days, injected with MNU intravenously (50 mg/kg body wt), and subsequently fed the control diet containing 2 mg folic acid/kg diet for 180 days. Glycine and succinylsulfathiazole (10 g/kg diet) were added to the diet to potentiate folate deficiency. Plasma folate concentrations were 15 ± 5, 77 ± 15 and 218 ± 47 ng/ml for the 0, 2 and 40 mg folic acid/kg diet groups at the time of MNU injection and 79 ± 8, 58 ± 6 and 56 ± 6 ng/ml at necropsy. Although the incidence of mammary cancer was not significantly different among the four groups,
cancer multiplicity was significantly lower in rats fed the 0 mg folic acid diet than those fed the 2 mg folic acid, the 40 mg folic acid or the 20 mg folic acid diets; there was no significant difference in cancer multiplicity among the latter three groups. Furthermore, the time required for 50% of the rats to develop palpable mammary tumors was significantly longer in the 0 mg folic acid group than in the 40 mg folic acid or the 20 mg folic acid group, but was not significantly different from that in the 2 mg folic acid group. Thus, Baggott’s study demonstrated that folate deficiency suppressed initiation and early promotion of MNU-induced mammary tumorigenesis (25).

As suggested by Baggott’s study (25), the inhibitory effect of folate deficiency on MNU-induced mammary tumorigenesis in rats may be a real effect on initiation and early promotion. However, it is possible that the conventional dose and route of MNU injection employed in the present study might have created an overwhelmingly carcinogenic milieu for folate status to modulate initiation of mammary tumorigenesis. Regardless of the levels of dietary folate, MNU probably induced and established neoplastic foci in mammary tissues. In this setting, folate deficiency probably suppressed the progression of and/or caused regression of established mammary neoplastic foci. This explanation is consistent with the biochemical function of folate. Interruption of folate metabolism in rapidly replicating neoplastic cells could cause ineffective DNA synthesis and hence the inhibition of tumor growth has been the basis of antitumor therapy using antifolate agents (49). Folate deficiency has been shown to induce regression and suppress progression of pre-existing neoplasms in experimental models (4,7,8,50±52). Therefore, it is possible that the inhibitory effect of folate deficiency on MNU-induced tumorigenesis in this rat model might have been primarily on promotion/progression of established mammary neoplastic foci. In this regard, although Baggott’s study was primarily designed to test the effect of folate on initiation and early promotion, it is possible that the observed effect of folate was actually on promotion/progression because of the dose and route of MNU employed in that study (25).

In the present study, dietary folate supplementation at four times the basal dietary requirement, which has consistently conferred protection against intestinal tumorigenesis in rodents in previous studies (4,7,8,27), did not inhibit mammary tumorigenesis. This level of dietary folate supplementation did not promote the progression of MNU-induced neoplastic foci in the present study in contrast to the promoting effect associated with this level of dietary folate supplementation on progression of established intestinal neoplastic foci observed in some studies (7,8). The lack of effect associated with folate supplementation on mammary tumorigenesis in the present study may be related to the fact that, in spite of significantly higher serum and hepatic folate levels, the mean mammary gland folate concentration of the folate-supplemented rats was not significantly different from that of the controls. Previous studies have demonstrated a dose-responsive tissue saturating effect of folate supplementation above four times the basal dietary requirement in rat colon (4), and the 8 mg folic acid diet has consistently induced significantly higher colonic mucosal folate concentrations compared with the 2 mg folic acid (control) diet in rodents (4,7,8,46,53). It is well known that different tissues express different folate requirements and hence different susceptibility to folate deficiency (40). Furthermore, folate accumulation in tissues is limited by the level of FPGS activity in the setting of substrate excess (47,48). FPGS catalyzes polyglutamation of intracellular folates, thereby allowing the retention of folate that would otherwise be lost because of efflux from the cell (47,48). Previous studies in animals and in cultured cells have shown that tissue levels of folate reach a plateau when FPGS is saturated from excess folate in the diet or culture medium (4,7,48). At present, there is no information in the literature regarding the levels of FPGS activity in normal mammary tissue. It is possible that the levels of FPGS activity in mammary gland are appreciably lower than the liver or colon and thus tissue folate is saturated at a much lower level of dietary folate in mammary gland compared with other tissues. However, it is also possible that higher levels of dietary folate supplementation above four times the basal dietary requirement may be necessary to increase mammary folate concentrations compared with the control diet.

One interesting finding in this study is that the extent of genomic DNA methylation is significantly lower in mammary adenocarcinomas than in non-neoplastic mammary tissues regardless of folate status. DNA methylation is an important epigenetic determinant in gene expression, in the maintenance of DNA integrity and stability, in chromatin modifications and in the development of mutations (29,30). Neoplastic cells simultaneously harbor widespread genomic DNA hypomethylation and more specific regional areas of hypermethylation (29,30). Genomic hypomethylation is an early, and consistent, event in carcinogenesis and is associated with genomic instability and increased mutations (29,30). Site-specific hypomethylation at the promoter region of tumor suppressor and mismatch repair genes is an important mechanism in gene silencing in carcinogenesis (29,30). Although promoter CpG islands hypermethylation and consequent inactivation of several tumor suppressor genes have been observed in human breast cancer (54), very few studies have reported genomic hypomethylation in human breast cancer (55,56). To our knowledge, our study is the first to demonstrate that genomic DNA hypomethylation is an epigenetic phenomenon associated with MNU-induced mammary tumorigenesis in rats. The extent of genomic DNA methylation in mammary adenocarcinomas and in non-neoplastic mammary tissues was not significantly modulated by folate status. This observation suggests that altered genomic DNA methylation was not a probable mechanism by which folate deficiency suppressed mammary tumorigenesis in our study. Because folate may modulate DNA methylation in a site-specific manner (43), however, the possibility that folate status may affect site-specific methylation of critical genes implicated in mammary tumorigenesis cannot be ruled out in the present study.

The strengths of the present study include: (i) the use of the amino acid-defined diet that is widely accepted as the standard means of inducing folate deficiency or providing supplemental dietary folate in rodents; (ii) the use of dietary levels of folate that have been shown to modulate development of other cancers in this strain of rats; (iii) measurements of systemic and mammary gland folate concentrations; (iv) rigorous histological confirmation of all mammary tumors to ensure an accurate determination of the rate of appearance and other tumor-specific parameters of adenomas and adenocarcinomas. However, several limitations associated with the present study need to be acknowledged. First, although the dose and route of MNU administration employed in the present study may be appropriate in studies examining the effect of other potential
chemopreventive agents in this model, the effect may be too overwhelmingly carcinogenic for folate to modulate. Therefore, the effect observed with dietary folate in the present study may be predominantly on promotion and progression, and not on initiation, of MNU-induced neoplastic foci. Secondly, the fat content of the diets used in the present study was higher than the AIN rodent diets that are more commonly used in experimental mammary tumor studies (10 versus 7% by weight). Animal studies have generally suggested that high fat diets enhance mammary tumorigenesis in rodents (57). Therefore, it is possible that the tumor-promoting effect associated with the higher fat content in our diets might have masked any modulating effect of dietary folate intervention. Thirdly, the mean mammary gland folate concentration associated with folate supplementation was not significantly higher than that of the control diet. Therefore, higher levels of folate supplementation above four times the basal dietary requirement may be necessary to significantly increase mammary gland folate concentrations and to observe any modulatory effect of folate supplementation on mammary tumorigenesis. Lastly, the number of animals employed in the present study did not allow us to achieve adequate statistical power. It would have required 103 animals in total to be 80% certain of detecting a 35% reduction in tumor incidence associated with folate deficiency compared with the control diet at a 5% level of significance.

In summary, our data suggest that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats. In contrast, dietary folate supplementation at four times the basal dietary requirement does not significantly modulate mammary tumorigenesis. Notwithstanding the limitations associated with this model, our data suggest that the role of folate in mammary tumorigenesis needs to be clarified in future studies for safe and effective prevention of breast cancer.

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