Effects of dietary folate on the development and progression of mammary tumors in rats

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Epidemiologic studies have suggested that dietary intake and blood levels of folate may be inversely related to the risk of breast cancer. However, epidemiologic evidence has not been consistent nor has it provided unequivocal support for this purported inverse relationship. Recent evidence has also raised a concern that folate supplementation provided during the promotion phase may promote carcinogenesis if provided after neoplastic foci are established in the target organ. This study investigated the effect of dietary folate deficiency and supplementation on the development and progression of mammary tumors in the N-methyl-N-nitrosourea (MNU) rat model. Weanling, female Sprague–Dawley rats were fed diets containing 0, 2 (control) or 8 mg folic acid/kg diet during the initiation or the promotion phase of MNU-induced mammary tumorigenesis. At necropsy, all macroscopic mammary tumors were identified and histologically confirmed. Dietary folate deficiency and supplementation provided during the initiation phase did not significantly modulate the development of mammary tumors. In contrast, dietary folate deficiency provided during the promotion phase significantly inhibited the rate of appearance, incidence, mean volume and weight of adenocarcinomas compared with the control and supplemental diets. Folate supplementation provided during the promotion phase did not significantly modulate mammary tumorigenesis compared with the control group. These data indicate that moderate folate deficiency inhibits, whereas dietary folate supplementation at four times the basal dietary requirement does not promote, the progression of MNU-induced mammary neoplastic foci in this rat model. However, the limitations associated with the route and dose of MNU administration preclude a definitive conclusion concerning the effect of folate status on the initiation of MNU-induced mammary tumorigenesis.

Introduction

Dietary intake and blood levels of folate, a water-soluble B-vitamin and an important co-factor in one-carbon metabolism (1), appear to be inversely associated with the risk of several malignancies including cancer of the colorectum, lungs, pancreas, esophagus, stomach, cervix and ovary, and neuroblastoma and leukemia (2,3). The precise nature and magnitude of the relationship between folate status and the risk of these malignancies, however, have not yet been clearly established (2,3). As an essential co-factor for the de novo biosynthesis of purines and thymidylate, folate plays an important role in DNA synthesis, stability and integrity, and repair, the aberrations of which have been implicated in carcinogenesis (4,5). Folate, in the form of 5-methyltetrahydrofolate, is also involved in remethylation of homocysteine to methionine, thereby ensuring the supply of S-adenosylmethionine (SAM), the primary methyl group donor for most biological methylation reactions including that of DNA (6). In this role, folate may modulate DNA methylation, which is an important epigenetic determinant in gene expression, in the maintenance of DNA integrity and stability, in chromosomal modifications and in the development of mutations (7). A growing body of evidence from in vitro, animal and human studies indicates that folate deficiency is associated with DNA strand breaks, impaired DNA repair, increased mutations and aberrant DNA methylation, and that folate supplementation can correct some of these defects induced by folate deficiency (2,4–6,8).

An accumulating body of epidemiologic studies also suggest an inverse association between folate status and the risk of breast cancer (9–23). However, 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 (24–29). Some epidemiologic studies have suggested that folate status alone may not be sufficient in modifying breast cancer risk. However, with alcohol consumption, a well-established risk factor for breast cancer development (30,31), folate deficiency potentiates, whereas folate supplementation reduces, the risk of breast cancer (12,14,15,19,20,22). Furthermore, some studies have suggested that folate status interacts with other dietary factors involved in one-carbon metabolism (e.g. methionine, vitamins B6 and B12) to modify breast cancer risk (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 C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene that encodes an enzyme critical for intracellular folate homeostasis (16,23,32–35).

Abbreviations: CpG, cytosine–guanine; MNU, N-methyl-N-nitrosourea.

Since only a few modifiable risk factors for breast cancer exist (31,36), recent epidemiologic observations that suggest that folate deficiency increases, whereas supplementation reduces, the risk of breast cancer underscore the need to further evaluate the role of folate in the development of this disease. Folate is generally regarded as safe and has long been presumed to be purely beneficial (37), and possesses biologically plausible mechanisms for cancer prevention (2,4–6,8,38). However, studies performed in animal models of colorectal cancer have shown that the dose and timing of folate intervention are critical in providing safe and effective chemoprevention; exceptionally high supplemental folate levels and folate intervention after microscopic neoplasia established in the colorectal mucosa promote rather than suppress colorectal carcinogenesis (39,40). These observations suggest that folate possesses dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention (39,40). Folate deficiency has an inhibitory effect, whereas folate supplementation has a promoting effect on the progression of established neoplasms (39,40). In contrast, folate deficiency in normal epithelial tissues appears to predispose them to neoplastic transformation, and modest levels of folate supplementation suppress, whereas supraphysiological doses enhance the development of tumors in normal tissues (39,40).

Given the dual modulatory role of folate in carcinogenesis, clarifying the potential role of folate in breast cancer prevention and progression in appropriate animal models is important because the vast majority of the US and Canadian populations are exposed to high amounts of folic acid owing to folic acid fortification and supplementation (40). One animal 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, but had no effect on tumor incidence, compared with a control and folate-supplemented diet (41). We have recently reported that dietary folate deficiency of a moderate degree (without anemia, growth retardation or premature death) suppresses, whereas a modest level (four times the basal dietary requirement) of folate supplementation does not significantly modulate, mammary tumorigenesis in the MNU rat model (42).

MNU is a direct acting, potent rodent mammary carcinogen that does not require metabolism (43). MNU induction is associated with a mammary tumor incidence of >90%, which are predominantly adenocarcinomas of a ductal origin with very few benign tumors (43). MNU is an alkylating agent that forms O6-methylguaine in DNA, leading to G→A transition during DNA replication (44). More than 85% of MNU-induced mammary tumors contain a G→A transition mutation at codon 12 of the Ha-ras oncogene, and consequent Ha-ras activation has been considered the primary mechanism by which MNU induces mammary tumors in rodent (45,46). MNU also induces DNA hypomethylation (47), and MNU-induced mammary tumors are associated with genomic DNA hypomethylation (42). Given the role of folate in DNA synthesis, mutagenesis and DNA methylation (2,4–6,8), folate possesses biologically plausible mechanisms by which it can modulate MNU-induced mammary tumorigenesis.

The aim of this study was to elucidate the effects of dietary folate deficiency and supplementation on the development and progression of mammary tumors by determining the effects of folate on the initiation and promotion phases of mammary tumorigenesis separately in the well-established MNU rat model of breast cancer. Our hypothesis was that dietary folate deficiency would enhance, whereas dietary folate supplementation would suppress, the initiation of MNU-induced mammary tumorigenesis. In contrast, once microscopic mammary neoplastic foci were established, we reasoned that dietary folate deficiency would suppress, whereas dietary folate supplementation would enhance, the promotion and progression of MNU-induced mammary tumorigenesis.

Materials and methods

Animals and dietary intervention

This study was approved by the Animal Care Committee of the University of Toronto. Specific pathogen-free, weanling female Sprague–Dawley rats (~50 g; Charles River Laboratories, St Constant, Quebec, Canada) were singly housed and maintained at 24 ± 2°C at 50% humidity with a 12 h light/dark cycle. Amino acid-defined diets (Dyets, Bethlehem, PA) (48) containing 0, 2 or 8 mg folic acid/kg diet were used. These diets constitute a standard method of inducing folate deficiency or providing supplemental dietary folate in rodents (48) and have been utilized extensively in previous studies of folate and colorectal cancer (49–52) and breast cancer (42). 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 (42,49). 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 (53). The diet containing 2 mg folic acid/kg is generally accepted as the basal dietary requirement for rodents (54). The diet containing 8 mg folic acid/kg represents folate supplementation four times the basal dietary requirement. This level of folate was chosen because an 8 mg/kg level has consistently provided a degree of chemoprevention against colorectal cancer in previous rodent studies (49,50,52). These diets contained 50 g cellulose/kg, 60% of the calories as carbohydrates, 23% as fat (or 10% by weight) and 17% as L-amino acids (48). The amount of methyl donors, methionine, choline and vitamin B12 were 8.2 g, 2.0 g and 50 µg per kg diet, respectively. The detailed composition of the diets has been published previously (48,52). Diets and water were provided ad libitum.

In the initiation study, rats (n = 61) were randomized to receive the diet containing 0 (n = 21), 2 (n = 20) or 8 (n = 20) mg folic acid/kg diet from weaning at 3 weeks of age for 5 weeks until 1 week following MNU injection, which occurred at 50 days of age. The initial diets were terminated 1 week after the MNU injection, and all the rats were placed on the control diet (2 mg folic acid/kg diet) for 22 weeks until the time of killing.

In the promotion study, rats (n = 92) were placed on the control diet (2 mg folic acid/kg diet) from weaning at 3 weeks of age until 5 weeks for continuous 1 week following MNU injection, which occurred at 50 days of age. One week following MNU administration, the rats were randomized to receive the diet containing 0 (n = 33), 2 (n = 30), or 8 (n = 30) mg folic acid/kg diet for 22 weeks until the time of killing.

MNU administration

At 50 days of age, all rats received one intraperitoneal injection of MNU (50 mg/kg body weight; Sigma Chemical, St Louis, MO). A single injection of 50 mg MNU/kg has become the standard dosage owing to its rapid induction and high incidence of mammary tumors combined with minimal toxicity and a short latency period of 3–6 months (55). MNU has a half-life of <1 h under physiological conditions (56) and its mutagenic effects (e.g. activated Ha-ras oncogene) have been shown to occur within a very short time after its administration (i.e. during the initiation of MNU-induced tumorigenesis) (57). During initiation, MNU forms adducts with DNA and cause mutations in the Ha-ras oncogenes that become fixed upon mitosis (58). During promotion, the initiated cells are stimulated to divide by mechanical, hormonal or genetic factors and form preneoplasias (58). Progression to a malignant phenotype with acquisition of metastatic capabilities is brought on by the increasing genetic instability of the tumor, leading to mutations at key genetic sites (58). There is unequivocal evidence that MNU-induced mammary tumorigenesis is initiated within 1 week after intraperitoneal injection (45,58,59). The experimental protocol of the MNU administration and the timing of dietary intervention used in the present study is the standard protocol that is designed to clarify the effect of a dietary factor on the initiation and promotion phases of MNU-induced mammary tumorigenesis (59) and has been successfully utilized in prior animal studies by our group (60,61).
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 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 drawn from the lateral tail vein of each rat within a week of MNU injection and from the heart at necropsy and centrifuged at 800 x g 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 MNU injection (i.p.) 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 (30 weeks of age). The liver from each rat was harvested, snap-frozen and stored at −70°C for the determination of hepatic folate concentration. All macroscopic mammary tumors were counted, excised and weighed, and the diameter of each tumor was recorded 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. (62) by two experienced pathologists (A.M. and R.R.) blinded to the study group independently. 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 mammary tissue folate determination.

Determination of folate concentration
Serum folate concentrations were determined by a standard microbiological microtiter plate assay using Lactobacillus casei. Serum folate concentrations were determined by a standard microbiological assay. The final preparations had a ratio of DNA extraction and mammary tissue folate determination. The size of DNA estimated by agarose-gel electrophoresis was 260 to A280 between 1.8 and 2.0. Interassay coefficients of variation for serum, hepatic and mammary tissue folate concentrations were 5, 5 and 6%, respectively. Interassay coefficients of variation for serum, hepatic and mammary tissue folate concentrations were 5, 5 and 7%, respectively.

DNA extraction
DNA from normal mammary tissue and mammary tumors was extracted by a standard technique using proteinase K followed by organic extraction (65). 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 A260 to A280 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 previously described (42,50,52). All analyses were performed in duplicate. Both intrassay and interassay coefficients of variation of the DNA methylation assay were 5%.

Statistical analysis
Between-group comparisons of continuous variables were assessed using the Kruskal–Wallis and Mann–Whitney non-parametric tests. For categorical response variables, differences among the groups were assessed by Pearson χ²-test. Differences in genomic DNA methylation between normal mammary gland and tumor in each diet group was assessed by the Wilcoxon signed ranks test. The Kaplan–Meier survival analysis and the Log rank test were used to compare the rates of tumor appearance among the three groups. Significance testing was set at P < 0.05 level (two-sided). Statistical analyses were performed using SPSS (version 10).

Results
Body weight and daily food consumption
Growth curves were not significantly different among the three dietary groups in both the initiation and promotion studies; at no time point did the mean body weights differ significantly among the three groups (data not shown). 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 (66). The mean daily food consumption, which was determined on a pre-assigned day of each week, was not significantly different among the three groups in both the initiation and promotion studies (data not shown).

Serum, liver and normal mammary gland folate concentrations
Initiation study. At the time of MNU injection (4 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 this time point were comparable to those observed in rats placed on the corresponding diets for 4–5 weeks in previous studies (42,49,50). This observation indicates 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 for the determination of the effect of folate status on the initiation phase of MNU-induced mammary tumorigenesis. At necropsy (22 weeks after beginning the control diet), the mean serum, hepatic and mammary gland folate concentrations of the three groups were not significantly different (Table I), and these levels were comparable with those observed in rats placed on the same diet for 24–27 weeks in previous studies (42,49,67).

Promotion study. At the time of MNU injection (4 weeks after beginning the control diet) the mean serum folate concentrations were not significantly different among the three groups (Table I). The mean serum folate concentrations of the three dietary groups at this time point were comparable to those observed in rats placed on the corresponding diets for 4–5 weeks in previous studies (42,49,50). At necropsy (22 weeks after the start of dietary intervention), the mean serum and hepatic folate concentrations were significantly different among the three dietary groups (P < 0.001; Table I) and were comparable to those observed in rats placed on corresponding diets for 24–27 weeks in previous studies (42,49,50). This observation indicates that a sufficient degree of systemic folate deficiency and supplementation was induced in the folate-deficient and -supplemented rats, respectively, after MNU injection for the determination of the effect of folate status on the promotion phase of MNU-induced mammary tumorigenesis. The mean mammary gland folate concentration of the folate-deficient group was significantly lower than the control and folate-supplemented groups (P ≤ 0.004; Table I). Although the mean mammary gland folate concentration of the folate-supplemented group was higher than that of the control group, this difference did not reach statistical significance (P = 0.063; Table I).

Effects of dietary folate on MNU-induced mammary tumorigenesis
No rat died prematurely nor was 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 euthanized rats was similar among the three groups (data not shown). Consistent with previous observations made in the MNU-Sprague–Dawley rat model of mammary tumorigenesis (42,43,58,59,62), > 90% of macroscopic mammary tumors in the present study were...
Table I. Serum, hepatic and mammary gland folate concentrations*  

<table>
<thead>
<tr>
<th>Diet group (n)</th>
<th>Initiation study</th>
<th>Promotion study</th>
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<tbody>
<tr>
<td></td>
<td>At the time of MNU injection</td>
<td>At necropsy (22 weeks of the 2 mg folic acid/kg diet)</td>
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<td></td>
<td>(4 weeks of dietary intervention)</td>
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<tr>
<td>Serum folate (ng/ml)</td>
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<tr>
<td>I (21)</td>
<td>21.7 ± 1.4a</td>
<td>50.6 ± 2.2b</td>
</tr>
<tr>
<td>II (20)</td>
<td>64.7 ± 2.5b</td>
<td>50.7 ± 1.9c</td>
</tr>
<tr>
<td>III (20)</td>
<td>107.8 ± 3.2d</td>
<td>53.6 ± 2.1f</td>
</tr>
<tr>
<td>Hepatic folate (µg/g tissue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (33)</td>
<td>3.2 ± 0.1a</td>
<td>6.8 ± 0.6c</td>
</tr>
<tr>
<td>II (30)</td>
<td>5.0 ± 0.2b</td>
<td>6.2 ± 0.4d</td>
</tr>
<tr>
<td>III (30)</td>
<td>12.9 ± 1.0e</td>
<td>12.7 ± 2.3f</td>
</tr>
<tr>
<td>Mammary folate (µg/g tissue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (33)</td>
<td>125.2 ± 10.6</td>
<td>141.0 ± 18.5</td>
</tr>
<tr>
<td>II (30)</td>
<td>114.0 ± 18.5</td>
<td>129.9 ± 10.2</td>
</tr>
<tr>
<td>III (30)</td>
<td>146.4 ± 13.3</td>
<td>110.3 ± 12.7</td>
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*Results are expressed as mean ± SEM. Means in a row with different letters at each time point significantly differ at P < 0.005 by between-group comparisons.

†In the initiation study, rats were randomized to receive the diet containing either 0 (I, folate deficiency), 2 (II, control, basal dietary requirement), or 8 (III, supplemented) mg folic acid/kg diet from weaning at 3 weeks of age for 5 weeks until 1 week following MNU injection. At 50 days of age, all the rats received an intraperitoneal injection of MNU. The initial diets were terminated 1 week after the MNU injection, and all the rats were placed on the control diet (2 mg folic acid/kg diet) for 22 weeks until the time of killing.

‡In the promotion study, rats were placed on the control diet (2 mg folic acid/kg diet) from weaning at 3 weeks of age for 5 weeks until 1 week following MNU injection. At 50 days of age, all the rats received an intraperitoneal injection of MNU. One week following MNU administration, the rats were randomized to receive the diet containing either 0 (I, deficient), 2 (II, control), or 8 (III, supplemented) mg folic acid/kg diet for 22 weeks until the time of killing.

In the initiation study, 100% of the tumors were adenocarcinomas in the folate-deficient group, whereas only identified histologically as adenomas (15%) or adenocarcinomas (85%) as either adenomas or adenocarcinomas. The analyses pertaining to mammary tumors were performed between the two study pathologists (kappa statistics = 0.95), and all the analyses were confined to adenocarcinomas alone (Table II). When the analyses were confined to adenocarcinomas alone, no significant difference in the final incidence of adenocarcinomas among the three dietary groups was observed (Table III). The mean tumor latency was not significantly different among the three dietary groups (Table III). The mean tumor latency was not significantly different among the three dietary groups (Table III). There was a non-significant trend toward lower tumor weight in the folate-deficient group compared with the control and folate-supplemented groups (Figure 1B). When the analysis was confined to adenocarcinomas alone, a significant difference in the final incidence of adenocarcinomas was observed between the two study pathologists (kappa statistics = 0.95). When the analysis was confined to adenocarcinomas alone, a similar pattern was observed in the final incidence of adenocarcinomas. The analyses pertaining to mammary tumors were performed between the two study pathologists (kappa statistics = 0.95), and all the analyses were confined to adenocarcinomas alone (Table II). When the analyses were confined to adenocarcinomas alone, no significant difference in the final incidence of adenocarcinomas among the three dietary groups was observed (Table III). The mean tumor latency was not significantly different among the three dietary groups (Table III). There was a non-significant trend toward lower tumor weight in the folate-deficient group compared with the control and folate-supplemented groups (Figure 1B).
Fig. 1. The rate of appearance of either mammary adenomas or adenocarcinomas (A) or adenocarcinomas alone (B) among the three dietary groups 
\(P\)-overall = 0.83 and \(P\)-overall = 0.81, respectively, by the Kaplan–Meier survival analysis) in the initiation study. In the initiation study, groups I, II and III received the 0 (deficient), 2 (control) and 8 (supplemented) mg folic acid/kg diet, respectively, from weaning at 3 weeks of age for 5 weeks until 1 week following MNU injection (at 50 days of age), followed by the 2 mg folic acid/kg diet for 22 weeks until the time of killing (30 weeks of age).

Fig. 2. (A) The rate of appearance of either mammary adenomas or adenocarcinomas among the three dietary groups \(P\)-overall = 0.10 by the Kaplan–Meier survival analysis) in the promotion study. (B) The rate of appearance of mammary adenocarcinomas among the three dietary groups \(P\)-overall = 0.02 by the Kaplan–Meier survival analysis; \(P = 0.01\) between groups I and II, \(P = 0.02\) between groups I and III and \(P = 0.87\) between groups II and III by the Log rank test) in the promotion study. In the promotion study, groups I, II and III received the 2 mg folic acid/kg diet (control) from weaning at 3 weeks of age for 5 weeks until 1 week following MNU injection (at 50 days of age), followed by the 0 (deficient), 2 (control) and 8 (supplemented) mg folic acid/kg diet, respectively, for 22 weeks until the time of killing (30 weeks of age).

Table II. Effects of dietary folate on the incidence, latency, multiplicity, volume and weight of mammary tumors in the initiation study

<table>
<thead>
<tr>
<th></th>
<th>Adenocarcinomas + adenomas</th>
<th>P-value ANOVA</th>
<th>Adenocarcinomas</th>
<th>P-value ANOVA</th>
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<tbody>
<tr>
<td>Diet group1</td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>I</td>
</tr>
<tr>
<td>Incidence (%)</td>
<td>68</td>
<td>70</td>
<td>70</td>
<td>0.99</td>
</tr>
<tr>
<td>Mean latency (weeks post-MNU injection)</td>
<td>18.4 ± 1.1</td>
<td>16.6 ± 1.0</td>
<td>17.5 ± 1.3</td>
<td>0.54</td>
</tr>
<tr>
<td>Mean multiplicity</td>
<td>1.6 ± 0.3</td>
<td>3.2 ± 1.8</td>
<td>1.1 ± 0.1</td>
<td>0.52</td>
</tr>
<tr>
<td>Mean volume (cm³)</td>
<td>4.0 ± 1.6</td>
<td>4.2 ± 1.8</td>
<td>2.0 ± 0.9</td>
<td>0.50</td>
</tr>
<tr>
<td>Mean weight (g)</td>
<td>1.5 ± 0.6</td>
<td>1.4 ± 0.5</td>
<td>1.0 ± 0.3</td>
<td>0.70</td>
</tr>
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</table>

1Results are expressed as mean ± SEM.

1Groups I, II and III received the 0 (deficient), 2 (control) and 8 (supplemented) mg folic acid/kg diet, respectively, from weaning at 3 weeks of age for 5 weeks until 1 week following MNU injection (at 50 days of age), followed by the 2 mg folic acid/kg diet for 22 weeks until the time of killing (30 weeks of age).
Table III. Effects of dietary folate on the incidence, latency, multiplicity, volume and weight of mammary tumors in the promotion study

<table>
<thead>
<tr>
<th>Diet group</th>
<th>P-value ANOVA</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Incidence (%)</td>
<td>61</td>
</tr>
<tr>
<td>Mean latency (weeks post-MNU injection)</td>
<td>17.9 ± 1.1</td>
</tr>
<tr>
<td>Mean multiplicity</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Mean volume (cm³)</td>
<td>0.9 ± 0.3³</td>
</tr>
<tr>
<td>Mean weight (g)</td>
<td>0.5 ± 0.1³a</td>
</tr>
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Results are expressed as mean ± SEM. Means in a row with different letters at each time point significantly differ at P < 0.04 by between-group comparisons.

Groups I, II and III received the 2 mg folic acid/kg diet (control) from weaning at 3 weeks of age for 5 weeks until 1 week following MNU injection (at 50 days of age), followed by the 0 (deficient), 2 (control) and 8 (supplemented) mg folic acid/kg diet, respectively, for 22 weeks until the time of killing (30 weeks of age).

Fig. 3. Effects of dietary folate on genomic DNA methylation in mammary adenocarcinomas and non-neoplastic mammary tissues as determined by the in vitro methyl acceptance assay in the initiation (A) and promotion (B) studies. The manner in which this assay is performed produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous ³H-methyl incorporation into DNA. Asterisk denotes significant differences by the Wilcoxon signed ranks test at P < 0.04 compared with adenocarcinomas within each dietary group. Values are mean ± SD.

87 and 86% of the tumors in the folate-deficient and control groups, respectively, were adenocarcinomas (Table II). Similarly, in the promotion study, 100% of the tumors were adenocarcinomas in both the folate-supplemented and control groups, whereas only 85% of the tumors were adenocarcinomas in the folate-deficient group (Table III). Although these observations were not statistically significant, these data suggest that folate deficiency might have retarded the progression of adenoma to adenocarcinoma.

Discussion

Our data from the initiation study indicate that dietary folate deficiency and supplementation do not significantly modulate the development of MNU-induced mammary tumors in this rodent model. The lack of effect of dietary folate deficiency on the initiation of MNU-induced mammary tumorigenesis is not entirely surprising because prior epidemiologic observations have suggested that folate deficiency alone may not be sufficient to modulate the development of breast cancer; folate deficiency increases the risk of breast cancer in those who consume alcohol regularly (12,14,15,19,20,22) and in those with genetic predispositions (e.g. MTHFR C677T polymorphism) (16,23,32–35). The lack of inhibitory effect associated with folate supplementation four times the basal dietary requirement may be related to the observation that this level of folate supplementation failed to significantly increase mammary tissue. However, for both the initiation (Figure 3A) and promotion (Figure 3B) studies, the degree of ³H-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.
Folate and breast cancer

Folate and breast cancer

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between blood measurements of folate and breast cancer risk has been equivocal in epidemiologic studies (22,29). Overall, the portfolio of epidemiologic evidence supporting the relationship between folate status and breast cancer risk is tenuous at best, although a clearer picture emerges when studies examining the joint effects of folate and alcohol are considered.

One interesting finding in the present 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. Neoplastic cells simultaneously harbor widespread genomic DNA hypomethylation and more specific regional areas of hypermethylation (7). Genomic hypomethylation is an early, and consistent, event in carcinogenesis and is associated with genomic instability and increased mutations (7). Site-specific hypermethylation at the promoter region of tumor suppressor and mismatch repair genes is an important mechanism in gene silencing in carcinogenesis (7). Although hypermethylation of promoter CpG islands and consequent inactivation of several tumor suppressor genes have been observed in human breast cancer (73), very few studies have reported genomic hypomethylation in human breast cancer (74,75). Our study demonstrates 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 likely mechanism by which folate deficiency suppressed mammary tumorigenesis in our study. Previous animal and in vitro studies have suggested that the effect of folate deficiency on DNA methylation depends on cell type, target organ and stage of transformation (6). The same degree of dietary folate deficiency and supplementation used in the present study also failed to induce significant changes in genomic DNA methylation in rodent liver and colon in previous studies (6). Since folate may modulate DNA methylation in a site-specific manner (6), 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.

MNU-induced mammary tumorigenesis in rat is different from the human disease in several important aspects: (i) the exposure of the mammary tissue to high dosages of the genotoxic chemical carcinogen as opposed to the natural etiologic cause involved in most cases of human breast cancer; (ii) the lack of p53 mutations, which is a common molecular event in human breast cancer development; and (iii) the primary carcinogenic mechanism via the ras-mediated signal transduction pathway, which is rarely involved in human breast cancer (43,58,59). Nonetheless, 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 initiation and promotion stages; and (iv) hormonally dependent mammary tumorigenesis (43,58,59).

In summary, our data suggest that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats, and this effect appears to be primarily via inhibition of the progression of established mammary neoplastic foci. In contrast, dietary folate supplementation at four times the basal dietary requirement does not significantly modulate mammary tumorigenesis in this model. However, the limitations associated with the route and dose of MNU administration preclude a definitive conclusion concerning the effect of folate status on the initiation of MNU-induced mammary tumorigenesis. Notwithstanding the limitations associated with this model, our data, in conjunction with the lack of convincing epidemiologic evidence for the protective effect of folate supplementation on breast cancer risk and with emerging evidence for the dual modulatory role of folate in carcinogenesis depending on the timing and dose of folate intervention (39,40), suggest that the role of folate in mammary tumorigenesis needs to be clarified in future studies.

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References


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