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Effect of folic acid supplementation on the progression of colorectal aberrant crypt foci

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Whether or not folic acid supplementation promotes the progression of colorectal preneoplastic lesions to cancer is an important public health issue, given mandatory fortification and widespread supplemental use of folic acid in North America. We investigated the effect of folic acid supplementation on the progression of aberrant crypt foci (ACF), the earliest precursor of colorectal cancer. Male Sprague-Dawley rats ($n = 152$) were placed on a control diet (2 mg folic acid/kg diet) at weaning and ACF were induced by azoxymethane. Six weeks post-ACF induction, rats were randomized to receive 0, 2, 5 or 8 mg folic acid/kg diet. At 34 weeks of age, rats were killed, and colorectal tumor parameters, plasma folate and homocysteine (a sensitive inverse indicator of tissue folate status) concentrations and rectal epithelial proliferation were determined. Although the number of ACF increased as dietary folic acid levels increased ($P = 0.015$), the incidence of colorectal tumors did not differ significantly among the four dietary groups. However, tumor multiplicity was positively correlated with dietary folic acid levels ($r = 0.32$; $P = 0.002$) and inversely with plasma homocysteine concentrations ($r = -0.32$; $P = 0.005$). Tumor burden was positively correlated with dietary folic acid levels ($r = 0.35$; $P = 0.001$) and plasma folate concentrations ($r = 0.33$; $P = 0.008$) and inversely with plasma homocysteine concentrations ($r = -0.42$; $P < 0.001$). Rectal epithelial proliferation was positively correlated with dietary folic acid levels ($r = 0.39$; $P < 0.001$) and plasma folate concentrations ($r = 0.34$; $P < 0.001$) and inversely with plasma homocysteine concentrations ($r = -0.37$; $P < 0.001$). Our data suggest that folic acid supplementation may promote the progression of ACF to colorectal tumors.

Introduction

The role of folate, a water-soluble B vitamin that is present in green leafy vegetables, asparagus, broccoli, Brussels sprouts, citrus fruit, legumes, dry cereals, whole grain, yeast, lima beans, liver and other organ meats, and folic acid, the synthetic form of folate that is used in supplements and fortified foods, in the development and progression of colorectal cancer is highly controversial at present. Dietary intake and blood measurements of folate have been shown to be inversely related to the risk of colorectal cancer or its precursor adenomas in most of the published observational epidemiologic studies (1) including two meta and pooled analyses (2,3). Evidence supporting this inverse association from epidemiologic studies using dietary folate has been more consistent than that from studies using total folate, possibly due to the incomplete or inadequate assessment of folic acid from foods and supplements by the lack of use of the composite 'dietary folate equivalents' (4). The portfolio of these epidemiologic studies suggest a 20–40% reduction in the risk of colorectal cancer or adenomas in subjects with the highest folate status compared with those with the lowest status (1–3). Small human intervention trials have also reported that folic acid supplementation (400 μg to 10 mg/day for 3 months to 2 years) improves or reverses several functional biomarkers of folate metabolism and colorectal cancer (1). While two folic acid chemoprevention trials (500 μg to 1 mg/day for 2–3 years) in subjects with previously resected colorectal adenomas did not demonstrate a protective effect of folic acid supplementation on the incidence of recurrent colorectal adenomas (5,6), one recent study reported that folic acid supplementation at 5 mg/day for 3 years significantly reduced the number of recurrent adenomas (7).

Recently, an emerging body of evidence has raised concern that high levels of folate/folic acid may in fact promote colorectal carcinogenesis. A recent population-based case–control study from Sweden has reported that high plasma folate concentrations are associated with an increased risk of colorectal cancer (8). Furthermore, the Aspirin/Folate Polyp Prevention Study (9) has reported that folic acid supplementation at 1 mg/day for 6 years significantly increased the recurrence of advanced colorectal adenomas by 67% and the incidence of multiple (≥3) colorectal adenomas by 2-fold compared with placebo in subjects with a history of colorectal adenomas. Furthermore, among those subjects who agreed to follow-up and continued the assigned treatment after the completion of the trial, folic acid supplementation was associated with a significant 2.6-fold increase in prostate cancer risk over 10 year follow-up (10).

The seemingly paradoxical effects of folate on colorectal cancer development and progression are not entirely surprising. Data from animal studies suggest that folate possesses dual modulatory effects on colorectal cancer development and progression depending on the timing and dose of folate intervention (1,11). In chemical carcinogen rodent models of colorectal cancer, a moderate degree of folate deficiency in the normal colorectum promoted the development of colorectal cancer, and modest levels of folic acid supplementation—up to 4× the basal dietary requirement (BDR) for rodents—suppressed, whereas supraphysiologic supplemental doses exceeding the BDR by 20–10 000× enhanced, the development of colorectal cancer (12–17). In two genetic models of intestinal tumors (Apc^{Min} and *Apc+/−xMsh2−/−*), moderate dietary folate deficiency enhanced, whereas modest levels of folic acid supplementation (4–10× the BDR) suppressed, the development and progression of adenomas, if folic acid intervention was started before the establishment of neoplastic foci in the intestine (18,19). If, however, folic acid intervention was started after the establishment of neoplastic foci, the same degree of dietary folate deficiency inhibited the progression and induced regression of the established neoplastic foci (18,19). A potential tumor-promoting effect of folic acid supplementation on the established neoplastic foci could not be clearly determined in these studies due to the inherent limitations associated with these genetic models (18,19). These observations suggest that folate status may have opposite effects on colorectal cancer development and progression depending on the timing of intervention (1,11).

Preclinical and human studies collectively suggest that folic acid supplementation may prevent the development of cancer in the normal colorectum (1,20). However, whether or not folic acid supplementation can promote the progression and growth of already existing preneoplastic colorectal lesions has not been unequivocally established yet. This is a critically important issue in the USA and Canada because dietary intake and blood measurements of folate in these countries have dramatically increased over the past decade owing to the introduction of mandatory folic acid fortification in 1998 (providing a daily average of 100–200 μg) (21,22) aimed at reducing the

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; BDR, basal dietary requirement; CI, confidence interval; RDA, recommended dietary allowance.

rate of neural tube defects. In addition, up to 30–40% of the North American population consume supplemental folic acid (400 μg in standard multivitamin or 1–5 mg in special preparations) (23) for several possible but as yet unproven health benefits. Given these considerations, we investigated whether folic acid supplementation can promote the progression of aberrant crypt foci (ACF) to colorectal adenocarcinoma in a well-established chemical carcinogen rodent model of colorectal cancer. ACF are the earliest recognizable putative preneoplastic lesions of colorectal cancer in rodents and humans based on the growth, morphological and molecular features (24,25).

Materials and methods

Animals and dietary intervention

This study was approved by the Animal Care Committee of the University of Toronto. Pathogen-free, 3-week-old male Sprague-Dawley rats ($n = 152$; ~50 g; Jackson Laboratory, Bar Harbor, ME) were placed on an amino acid-defined diet containing 2 mg folic acid/kg diet (control diet; Dyets, Bethlehem, PA) until 10 weeks of age. The diet containing 2 mg of folic acid/kg diet is generally accepted as the BDR for rats (26) and was selected to represent the recommended dietary allowance (RDA) for humans, which is 400 μg/day of dietary folate equivalents (27). Starting at 5 weeks of age, all rats were given two weekly subcutaneous injections of azoxymethane (AOM) (15 mg/kg body wt; Midwest Research Institute, Kansas City, MO). Four weeks after the last AOM treatment (i.e. 10 weeks of age or 7 weeks on the control diet), the animals were randomized to receive the amino acid-defined diet containing 0 ($n = 36$), 2 ($n = 44$), 5 ($n = 36$) or 8 ($n = 36$) mg folic acid/kg diet ($n = 35$/group) for the next 24 weeks until the time of killing (i.e. 34 weeks of age).

Amino acid-defined diets containing different levels of folic acid constitute a standard method of inducing folate deficiency and supplemental dietary folate in rodents (28) and have been extensively used in previous studies of dietary folate and cancer in rodents (12,13,18,19,29–31). The diet containing 0 mg folic acid/kg diet produces progressive folate deficiency of a moderate degree through weeks 3–5, after which systemic folate indicators stabilize (12,19,29–31). Although this diet is completely devoid of folate, severe folate deficiency is not induced due to *de novo* synthesis of folate by intestinal bacteria, some of which is incorporated into the tissue folate of the host (32), and animals survive over 30 weeks without anemia, growth retardation or premature death (29–31). This degree of moderate folate deficiency has been shown to enhance the development of colorectal cancer in previous rodent studies (12,13,18,19). The diet containing 5 mg folic acid/kg (2.5× BDR) represents the probable average postfortification total folate intake of ~800 to 1000 μg/day in North American populations who consume multivitamins containing 400 μg folic acid (33). The diet containing 8 mg folic acid/kg (4× BDR) was chosen because this supplemental level had consistently provided chemoprevention against colorectal cancer in previous rodent studies (12,13,18,19,31). It corresponds to ~1.6 mg folic acid/day in humans, reflecting the level that might be consumed by a subgroup of the North American population who receive 1 mg folic acid/day for certain medical conditions in the postfortification era (33). The rat 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 (28). The detailed composition of the diets has been published previously (19,28). Diets and water were provided *ad libitum*. Body weights were recorded weekly. The daily food consumption of each group was measured on a predetermined day of each week.

AOM induction of colorectal ACF and adenocarcinomas

Rats were given two weekly subcutaneous injections of AOM (15 mg/kg body wt) at 5 weeks of age according to the standard protocol (34). Microscopic adenocarcinomas develop between 12 and 18 weeks post-AOM treatment, and by 18–24 weeks, macroscopic adenocarcinomas are present in the majority of animals (34). About 85% of AOM-treated rats develop colon tumors, with a mean of three tumors per rat: ~70% are adenocarcinomas and the rest are adenomas (34). Most of the adenocarcinomas are well differentiated, invading into submucosal and muscular layers and some metastasize to regional lymph nodes and liver (34). AOM-treated rats develop ACF as early as 2 weeks but usually within 4–6 weeks of AOM treatment (14,16,17,34). Based on this time course of colorectal ACF and adenocarcinoma development post-AOM treatment, we chose to start the dietary intervention with four different levels of folic acid 4 weeks after the last AOM treatment, the time point at which ACF are established in the colorectum, in order to test the potential tumor-promoting effect of folic acid supplementation on ACF. Prior to dietary intervention, we confirmed the presence of ACF in seven sentinel rats at this time point. We chose to kill the remaining animals at 28 weeks after the last AOM injection, the time point at which the majority of the AOM-treated animals have developed colorectal adenocarcinomas.

The AOM rat model is a well-established animal model of colorectal cancer that is similar to human colorectal cancer in many clinical (propensity for distal colon), histopathological (development of ACF, adenomas and invasive adenocarcinomas) and molecular genetic (mutations of the *Apc* tumor suppressor gene and of the *K-ras* and *β-catenin* proto-oncogenes; mismatch repair defects; Cox-2 overexpression) aspects (35). However, AOM-induced colorectal cancer is different from human colorectal cancer in several aspects, including lack of *p53* mutations and exposure of the colon to high dosages of the genotoxic chemical carcinogen as opposed to the natural etiology involved in most sporadic cases of human colorectal cancer (35). Notwithstanding these limitations, this model has been extensively used in studies that investigated the effects of nutritional factors, including folic acid (14,16,17), and potential chemopreventive agents on the development and progression of colorectal cancer because it is a simple, reproducible and effective model (35–37). Furthermore, this rodent model accurately predicts the effects of these nutritional and chemopreventive agents on colorectal cancer in humans (36).

Sample collection and analysis of colorectal ACF and tumors

The rats were killed by carbon dioxide inhalation followed by cervical dislocation at 28 weeks after the last AOM injection (34 weeks of age). At necropsy, blood was drawn from the heart and centrifuged at 25 000*g* for 10 min at 4°C. Plasma was stored at −70°C with and without 0.5% ascorbic acid for plasma folate and homocysteine assays, respectively. The liver from each rat was harvested, snap-frozen and stored at −70°C for determination of hepatic folate concentrations. The entire colorectum, from the cecum to the anus, from each animal was immediately excised, put on a glass plate suspended on crushed ice, opened longitudinally and flushed with phosphate-buffered saline solution to eliminate fecal debris. The entire length of the colorectum was examined in a blinded fashion for any macroscopic lesions. All macroscopic lesions were excised, fixed in formalin and processed in a standard manner for hematoxylin and eosin staining for histological confirmation of adenomas and adenocarcinomas by a gastrointestinal pathologist (A.M.) blinded to the study groups. The longest diameter of each macroscopic lesion was measured and recorded. The rectum from each animal (the distal 2 cm of the excised colorectum) was cut, placed in a cassette with a foam cushion and stored in 10% neutral buffered formalin for the determination of epithelial proliferation. The remaining length of the colon was laid flat between two pieces of Whatman filter paper in 10 cm tissue culture dishes and fixed in Bouin's solution for subsequent ACF enumeration. The fixed colon was then placed on a microscope slide with the mucosal side up and assessed for ACF using a light microscope (Nikon, Labophot, Japan) at ×40 magnification by a single observer (F.D.) blinded to the study groups. ACF were identified by their dark staining, larger size, increased pericryptal space and their slight elevation above the mucosal surface according to the previously published criteria (38). The total number of ACF and crypt multiplicity (number of crypts per focus) were determined for each colon.

Determination of folate and plasma concentrations

Plasma folate concentrations were determined by a standard microbiological microtiter plate assay using *Lactobacillus casei* (39). Hepatic folate concentrations were determined by the same microbiological assay (39), utilizing a previously described method for tissue folate extraction and conjugase treatment (40). Total plasma homocysteine concentrations were determined using the Axis™ Homocysteine EIA kit (Abbott Laboratories, Mississauga, Ontario, Canada) according to the manufacturer's protocol as described (41).

Epithelial proliferation

Epithelial proliferation of the rectum was determined by staining histological sections with monoclonal antibodies against Ki-67, a nuclear protein expressed in proliferating cells, as described previously (42). Ki-67-staining index was expressed as a percent of positively stained nuclei, in relation to the total number of cells considered, and a scoring index as used to group range of percentages are as follows: 0 to <1%; 1+ to 2–5%; 2+ to 6–10%; 3+ to 11–20% and 4+ to >21%.

Statistical analysis

Based on the tumor incidence data from our previous animal experiment using the chemical carcinogen rat model colorectal cancer (13) and an assumption of a linear dose response, a sample size of 35 per each group provided 80% power to detect a small change in the probability of developing at least one tumor (an odds ratio of 1.5 for each increase in dietary folate) at the 0.05 level of significance.

The effect of dietary folate on continuous outcomes was tested using one-way analysis of variances. Tukey's method was used to adjust for multiple

post-hoc comparisons. Correlations between continuous dependent variables were tested using the non-parametric Spearman's correlation test. Associations between categorical variables were analyzed using Pearson's χ^2 test, associations between ordinal variables were analyzed using the Mantel–Haenszel test for linear association. Rat body weight was analyzed using ordinary least squares regression, taking into account the repeated measures nature of the data. Statistical analyses were performed using SPSS 14.0 for Windows (SPSS, Chicago, IL). Results are expressed as mean ± SEM. All significance tests were two sided and were considered significant at $P < 0.05$.

Results

Body weight, plasma folate and homocysteine and hepatic folate concentrations

Growth curves were not significantly different among the four dietary groups; at no time point did the mean body weights differ significantly among the four dietary folate groups.

The mean plasma folate concentrations were significantly different among the four dietary groups ($P < 0.001$; Table I), increasing with increased dietary folic acid levels up to 5 mg/kg diet (Table I). The mean plasma folate concentration of the rats fed 5 mg folic acid/kg diet was not significantly different from that of those fed 8 mg folic acid/kg diet (Table I).

The mean hepatic folate concentrations were also significantly different among the four dietary groups ($P = 0.002$; Table I). The rats on the 0 mg folic acid/kg diet had significantly lower hepatic folate concentrations than those receiving 5 or 8 mg folic acid/kg diet ($P < 0.03$; Table I) and the rats on the 2 mg folic acid/kg diet had the value intermediate between these groups (Table I). Hepatic folate concentrations were significantly correlated with plasma folate concentrations ($r = 0.42$; $P < 0.001$).

The mean plasma concentration of homocysteine [an accurate inverse functional indicator of folate status (43)] in the group receiving 0 mg folic acid/kg diet was significantly higher, by a factor of 3.6- to 5.1-fold, than the mean values of the three groups receiving the BDR or supplemental levels of folic acid ($P < 0.001$; Table I). The mean plasma homocysteine concentrations among the three groups receiving different levels of dietary folic acid were not significantly different (Table I). This is consistent with previous studies that have suggested that folic acid supplementation does not lower plasma homocysteine concentrations beyond the level achieved by dietary folate at the daily requirement (13,44). Plasma homocysteine concentrations correlated inversely with both plasma ($r = -0.64$; $P < 0.001$) and hepatic ($r = -0.40$; $P < 0.001$) folate concentrations.

Effect of folic acid supplementation on colorectal ACF

The total number of colonic ACF increased as dietary folic acid levels increased ($P = 0.003$; Table II). Between group comparisons indicated that the total number of colonic ACF was significantly higher, by 54%, in the rats receiving the 8 mg folic acid/kg diet than in the rats receiving the 0 mg folic acid/kg diet ($P = 0.011$; Table II). Although the total number of colonic ACF was 22–36% higher in the rats receiving the 8 mg folic acid/kg diet than in the rats receiving the 2 and 5 mg folic acid/kg diets, this did not reach statistical significance ($P = 0.07$ and $P = 0.36$, respectively; Table II). The mean number of crypts per focus was also significantly higher, by 15–16%, in the rats receiving the 8 mg folic acid/kg diet than in the rats receiving the 0 and 5 mg folic acid/kg diets ($P < 0.04$; Table II).

Effect of folic acid supplementation on colorectal tumor incidence

Fifty-six to 61% of the animals developed colorectal adenomas, adenocarcinomas or both. The incidence of colorectal tumors did not differ significantly among the four folic acid groups (Figure 1), nor did the incidence of colorectal adenomas or of adenocarcinomas as the most advanced neoplastic lesion (Figure 1). However, there was a linear increase, albeit not statistically significant, in the incidence of colorectal tumors (adenomas + adenocarcinomas) as dietary levels of folic acid increased (Figure 1).

Effect of folic acid supplementation on colorectal tumor multiplicity

In tumor-bearing rats, the mean number of colorectal tumors per rat did not differ significantly among the four dietary folic acid groups (Table II), nor did the mean number of colorectal adenomas per rat and of colorectal adenocarcinomas per rat, analyzed separately (Table II). However, the mean number of colorectal tumors per tumor-bearing rat was significantly correlated with dietary folic acid levels ($r = 0.33$; $P = 0.002$) but not with plasma ($r = 0.2$; $P = 0.11$; Figure 2A) or liver ($r = 0.24$; $P = 0.1$) folate concentrations. The

Table I. Effect of dietary folic acid supplementation on plasma and hepatic folate and plasma homocysteine concentrations[a]

Dietary folic acid level (mg/kg diet) (*n*)	0 (36)	2 (44)	5 (36)	8 (36)	*P* analysis of variance
Plasma folate concentration (ng/ml)	6.0 ± 1.3[b]	32.2 ± 1.8[c]	72.8 ± 2.8[d]	78.2 ± 3.8[d]	<0.001
Hepatic folate concentration (μg/g tissue)	5.0 ± 0.3[b]	7.7 ± 0.3[b,c]	8.5 ± 0.3[c]	9.9 ± 0.3[c]	0.002
Plasma homocysteine concentration (μmol/l)	21.9 ± 1.3[b]	5.9 ± 0.2[c]	6.0 ± 0.2[c]	4.3 ± 0.3[c]	<0.001

[a]Values are mean ± SEM. Within a row, means that share the same superscript letter do not differ significantly at the 0.001 level (after applying Tukey's adjustment for multiple comparisons).

Table II. Effect of dietary folic acid supplementation on colorectal ACF, adenomas and adenocarcinomas and rectal epithelial proliferation[a]

Dietary folic acid level (mg/kg diet) (*n*)	0 (36)	2 (44)	5 (36)	8 (36)	*P* analysis of variance	*P* for trend
Mean number of colorectal ACF	84.6 ± 7.3[b]	93.4 ± 6.6[b,c]	108.1 ± 8.7[b,c]	137.9 ± 10.1[c]	0.015	0.003
Mean number of crypts per focus	3.4 ± 0.1[b]	3.5 ± 0.1[b,c]	3.4 ± 0.1[b]	3.9 ± 0.1[c]	0.019	0.06
Mean number of colorectal tumors per tumor-bearing animal	2.8 ± 0.3	5.2 ± 0.6	5.0 ± 1.3	5.7 ± 0.8	0.19	0.002
Mean number of colorectal adenocarcinomas per tumor-bearing animal	1.2 ± 0.1	1.8 ± 0.2	1.9 ± 0.4	2.2 ± 0.3	0.12	0.006
Mean sum of colorectal tumor diameters per tumor-bearing animal (cm)	0.5 ± 0.1[b]	1.2 ± 0.2[b,c]	1.3 ± 0.3[b,c]	1.6 ± 0.4[c]	0.042	0.001
Rectal epithelial proliferation (%)	7.0 ± 0.4[b]	7.6 ± 0.5[b,c]	8.8 ± 0.5[c,d]	9.6 ± 0.4[d]	0.001	<0.001

[a]Values are mean ± SEM. Within a row, means that share the same superscript letter do not differ significantly at the 0.05 level (after applying Tukey's adjustment for multiple comparisons).

mean number of colorectal tumors per tumor-bearing rat was significantly inversely correlated with plasma homocysteine concentrations ($r = -0.32$; $P = 0.005$; Figure 2B). Similarly, the mean number of colorectal adenocarcinomas per tumor-bearing rat was significantly correlated with dietary folic acid levels ($r = 0.32$; $P = 0.006$) but not with plasma ($r = 0.21$; $P = 0.12$) or liver ($r = 0.24$; $P = 0.09$) folate concentrations. The mean number of colorectal adenocarcinomas per tumor-bearing rat was significantly inversely correlated with plasma homocysteine concentrations ($r = -0.29$; $P = 0.017$). The number of colorectal adenomas per tumor-bearing rat was not significantly correlated with dietary folic acid levels, plasma or liver folate concentrations or plasma homocysteine concentrations.

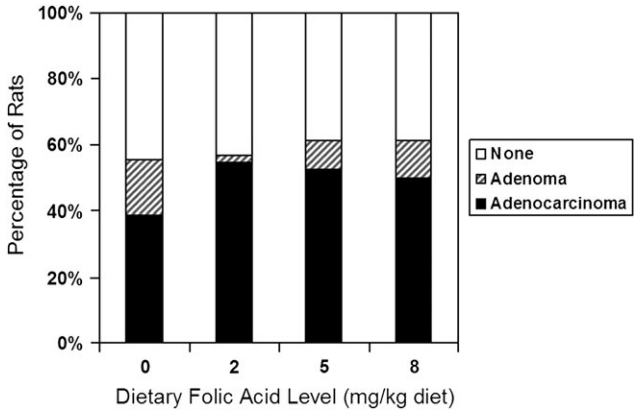

Fig. 1. Effect of folic acid supplementation on the incidence of the most advanced grade of colorectal neoplastic lesion.

Effect of folic acid supplementation on colorectal tumor burden

The sum of colorectal tumor diameters per tumor-bearing rat is used as a proxy of tumor burden. The mean sum of colorectal tumor diameters per tumor-bearing rat was significantly different among the four dietary folic acid groups ($P = 0.042$; Table II). The mean sum of colorectal tumor diameters per tumor-bearing rat was significantly correlated with dietary folic acid levels ($r = 0.35$; $P = 0.001$) and with plasma folate concentrations ($r = 0.33$; $P = 0.008$; Figure 3A) but not with liver folate concentrations ($r = 0.13$; $P = 0.38$). The mean sum of colorectal tumor diameters per tumor-bearing rat was significantly inversely correlated with plasma homocysteine concentrations ($r = -0.42$; $P < 0.001$; Figure 3B).

Effect of folic acid supplementation on rectal epithelial proliferation

The results of the analyses of rectal epithelial proliferation using percentages of Ki-67-stained cells and the scoring index of group

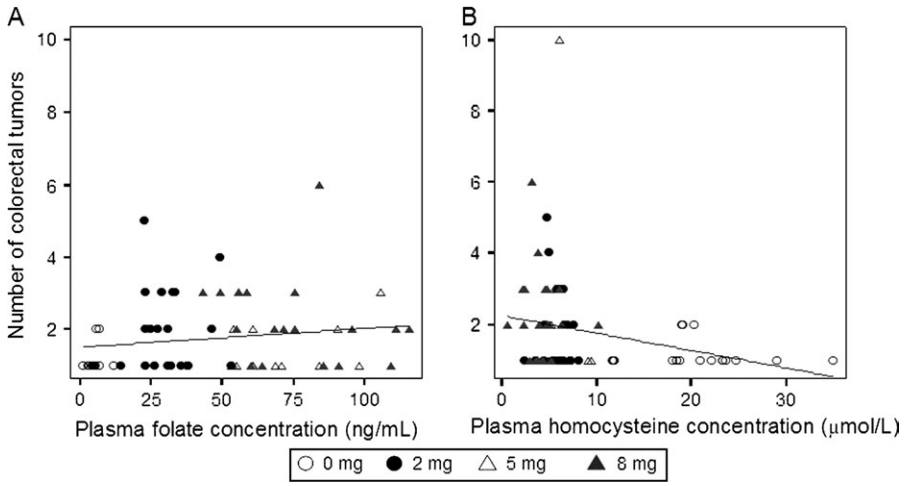

Fig. 2. (**A**) Correlations between the mean number of colorectal tumors (adenomas + adenocarcinomas) per tumor-bearing rat and plasma folate concentrations ($r = 0.2$; $P = 0.11$). (**B**) Correlations between the mean number of colorectal tumors and plasma homocysteine concentrations ($r = -0.32$; $P = 0.005$).

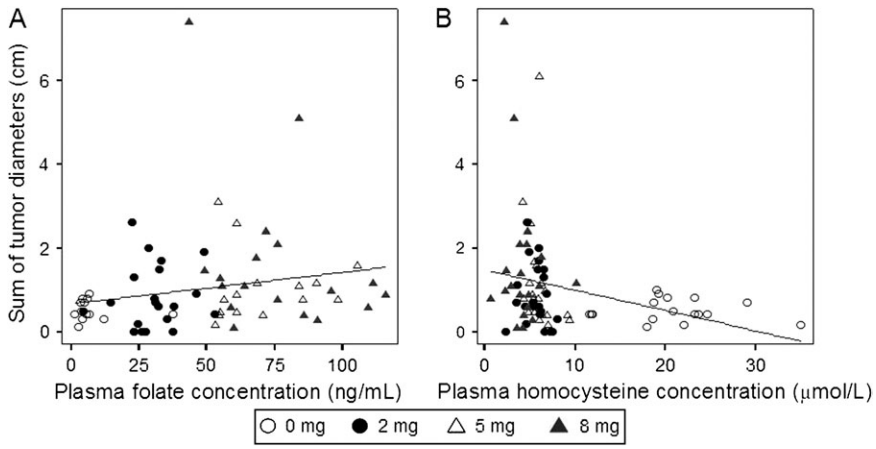

Fig. 3. (**A**) Correlations between the mean sum of colorectal tumor diameters per tumor-bearing rat and plasma folate concentrations ($r = 0.33$; $P = 0.008$). (**B**) Correlations between the mean sum of colorectal tumor diameters per tumor-bearing rat and plasma homocysteine concentrations ($r = -0.42$; $P < 0.001$).

range of percentages were similar, and hence, the data using percentages of Ki-67-stained cells are presented. Rectal epithelial proliferation was significantly different among the four dietary groups ($P = 0.001$; Table II; Figure 4). Rectal epithelial proliferation was significantly correlated with dietary folic acid levels ($r = 0.39$; $P < 0.001$) and with plasma folate concentrations ($r = 0.34$; $P < 0.001$; Figure 5A) and significantly inversely correlated with plasma homocysteine concentrations ($r = -0.37$; $P < 0.001$; Figure 5B). In tumor-bearing rats, rectal epithelial proliferation was significantly correlated with the mean number of colorectal tumors ($r = 0.23$, $P = 0.034$) and with the mean number of colorectal adenocarcinomas ($r = 0.28$, $P = 0.02$). However, rectal epithelial proliferation was not significantly correlated with the mean sum of colorectal tumor diameters in tumor-bearing animals.

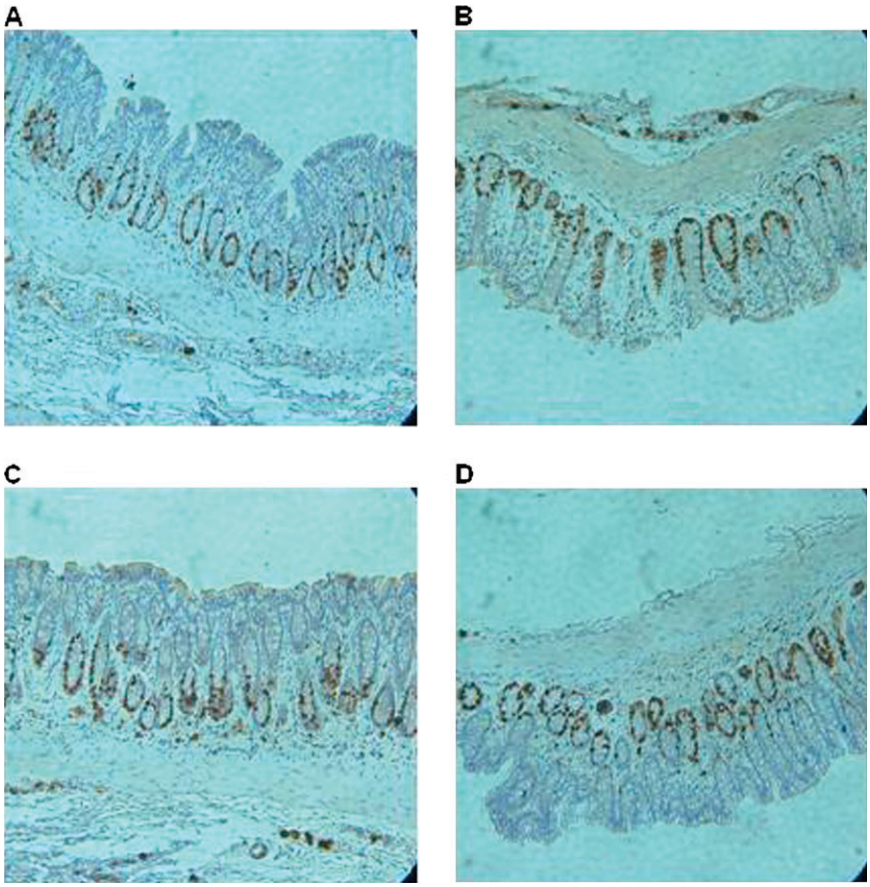

Fig. 4. Representative images of ki-67 stained rectal epithelial cells from four dietary groups containing (**A**) 0 mg folic acid/kg diet (4% positive stained nuclei in relation to the total number cells considered). (**B**) Two milligram folic acid/kg diet (8%). (**C**) Five milligram folic acid/kg diet (11%) and (**D**) 8 mg folic acid/kg diet (17%).

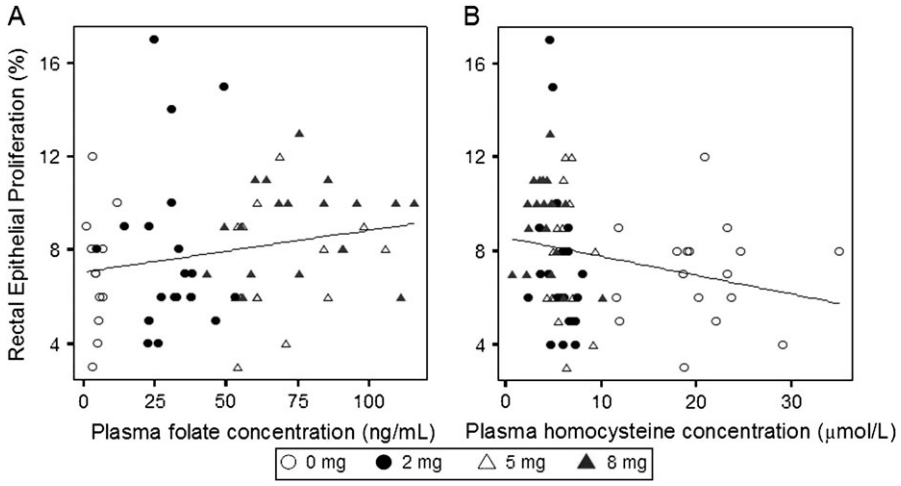

Fig. 5. (**A**) Correlations between rectal epithelial proliferation and plasma folate concentrations ($r = 0.34$; $P < 0.001$). (**B**) Correlations between rectal epithelial proliferation and plasma homocysteine concentrations ($r = -0.37$; $P < 0.001$).

Discussion

The growing body of evidence from preclinical studies suggests that folate plays a dual role in colorectal cancer development and progression depending on the timing of intervention (1,20). In the normal colorectum, folate deficiency appears to enhance, whereas folic acid supplementation suppresses, the development of colorectal cancer (1,20). In contrast, once preneoplastic foci are established, folate deficiency inhibits the progression and induces regression of these established preneoplastic foci (1,20). However, whether or not folic acid supplementation can facilitate the progression and growth of preneoplastic lesions in the colorectum has not yet been clearly demonstrated in preclinical studies. In the present study, we specifically tested whether folic acid supplementation could promote the progression of ACF—the earliest recognizable putative preneoplastic lesions of colorectal cancer in rodents and humans (24,25)—to colorectal adenocarcinoma in the well-established AOM rat model of colorectal cancer. Our *a priori* hypothesis was that folic acid supplementation would promote the progression of ACF to adenocarcinomas.

Although not uniformly consistent, our data collectively support our hypothesis and suggest that folic acid supplementation indeed seems to promote the progression of colorectal ACF. Folic acid supplementation incrementally increased the total number of ACF and the mean number of crypts per focus. Although the incidence of colorectal tumors was not significantly different among the four dietary groups with increasing folic acid levels, there was a linear increase, albeit not statistically significant, in the incidence of colorectal tumors as dietary levels of folic acid increased. A post-hoc analysis, based on the effect size observed in the current study, revealed that 382 and 734 rats in total would have been required to show that folic acid supplementation has a significant modulatory effect on the incidence of colorectal adenomas and adenocarcinomas, respectively. Therefore, the sample size used in the present study, based on an *a priori* calculation, was insufficient to demonstrate an unequivocal tumor-promoting effect of folic acid supplementation on colorectal tumor incidence. The tumor-promoting effect of folic acid supplementation was supported by the significant positive correlations between dietary folic acid levels and the multiplicity and tumor burden of colorectal tumors and by the significant inverse correlations between plasma concentrations of homocysteine and the same parameters of colorectal tumors. The correlations between plasma folate concentrations and tumor parameters were less consistent probably because of the fact that plasma folate concentrations reflect short-term, but not long-term, folate status. One interesting observation, albeit non-significant, is that while the rats on the 0 mg folic acid diet had a higher incidence of colorectal adenomas than the three folic acid-supplemented dietary groups, the incidence of colorectal adenocarcinomas was ~25% lower than that seen in the three folic acid-supplemented groups (Figure 1). This suggests that while mild to moderate folate deficiency might have enhanced the progression of ACF to adenomas, the same degree of folate deficiency might have suppressed the progression of adenomas to adenocarcinomas.

We did not measure colonic mucosal folate concentrations in the present study for the following reasons: (i) the entire colon was processed and fixed in Bouin's solution for subsequent ACF enumeration after harvesting all macroscopic tumors; once fixed in Bouin's solution, colonic mucosal folate concentrations cannot be determined and (ii) the AOM-induced colon harbored numerous ACF (means ranging from 86 to 132) and hence, folate concentrations in 'non-neoplastic' colonic mucosa could not be determined. Nevertheless, previous animals studies using the same diets (ranging from 0 to 8 mg folic acid/kg diet) and rats have demonstrated strong direct correlations between colonic mucosal folate concentrations and dietary and plasma folate concentrations and inverse correlations between colonic mucosal folate concentrations and plasma homocyteine concentrations (13,45). In this regard, plasma homocysteine concentrations were shown to be the best indicator of colonic mucosal folate concentrations (46,47). In the present study, the most consistent correlations with the selected tumor parameters were observed for plasma homocysteine concentrations.

We deliberately selected two modest supplemental levels of folic acid in the present study to reflect two representative intake levels of folate in the North America in the postfortification era. The average total folate intake postfortification is estimated to be ~400 μg/day in supplemental non-users, of which ~200 μg/day is in the form of folic acid provided in enriched products (48). For those taking multivitamins containing folic acid, the estimated total intake is ~800 μg/day (48). However, these estimated folate intake are probably to be underestimates. Several studies have suggested that postfortification folate intake in the USA population may be about twice that originally anticipated (33). Furthermore, it has been estimated that the intake of synthetic folic acid can easily exceed the recommended upper intake level of 1000 μg/day (27) due to use of multivitamins (400 μg), health drinks or bars (up to 400 μg), breakfast cereals (up to 400 μg/serving) and folic acid fortification (~100 to 200 μg/day) (4). Higher supplemental levels of folic acid (1–5 mg/day) are routinely provided to certain patients on antifolate medications to prevent adverse effects relating to folate depletion (4). Given these considerations, we selected 5 mg folic acid/kg (2.5× BDR) to represent the probable average postfortification total folate intake in the North American population consuming fortified food and other products, naturally folate-rich food and multivitamins containing 400 μg folic acid; this level probably approaches the daily recommended upper intake level of folate in North America [1000 μg/day; 2.5× the RDA (27). The diet containing 8 mg folic acid/kg (4× BDR) was chosen because this supplemental level corresponds to ~1.6 mg folic acid/day in humans (4× RDA or 1.6× the recommended upper intake level), which reflects the level that might be consumed by a subgroup of the North American population receiving 1 mg folic acid/day for certain medical conditions in the postfortification era. Furthermore, this supplemental level had consistently provided a chemopreventive effect against colorectal cancer in previous rodent studies (12,13,18,19,31). Because of inherent differences in folate metabolism between humans and rats, our selected dietary folic acid levels may not accurately reflect the corresponding levels in humans (49). Folic acid supplementation at 2.5× and 4× BDR significantly increased plasma folate concentrations by 2.3- and 2.4-folds, respectively, compared with the control diet containing the BDR amount folic acid in the present study (Table I). In human intervention trials, folic acid supplementation at 2–3× RDA increased plasma folate concentrations by 3- to 7-folds compared with controls (9,50,51). Interestingly, in human intervention trials, folic acid supplementation at 6–12.5× RDA increased plasma folate concentrations only by 2- to 7-folds compared with controls (52,53).

Some of the purported adverse effects of high levels of folate, including the potential tumor promoting effect, have been attributed to folic acid (49,54). Folic acid is not found in nature nor is it a normal metabolite. It must be reduced, first to dihydrofolate and then to tetrahydrofolate by dihydrofolate reductase and methylated to 5-methyltetrahydrofolate (the predominant folate found in blood), in the liver and to a lesser degree in the intestine, before it can enter the folate cycle (49). Recent evidence suggests that rats, unlike humans, have a comparatively high dihydrofolate reductase activity (49). Consequently, rats would have to be orally dosed with a much greater than *pro rata* amount of folic acid in order to elicit the same circulating plasma concentrations of folic acid in humans in order to assess the impact of systemic exposure of folic acid (49). Therefore, the two supplemental levels of dietary folic acid in rats probably achieved much lower plasma concentrations of folic acid than would have been achieved by the equivalent supplemental levels of folic acid in humans. Nevertheless, these modest supplemental levels of folic acid appear to exert the tumor-promoting effect on colorectal ACF in rats. It would be interesting to determine whether higher supplemental levels of folic acid demonstrate a greater magnitude of and more consistent and unequivocal evidence for the tumor-promoting effect of folic acid supplementation.

As an essential cofactor for the *de novo* biosynthesis of thymidylate and purines, folate plays a critical role in DNA synthesis, stability, integrity and repair (55,56). Mechanistically, the most probable

mechanism by which folic acid supplementation may promote the progression of established preneoplastic lesions in the colorectum is the provision of nucleotide precursors to rapidly replicating preneoplastic cells for accelerated proliferation and progression (1,20). Our data indeed demonstrate that rectal epithelial proliferation is significantly positively correlated with both dietary folic acid levels and plasma folate concentrations and is significantly inversely correlated with plasma homocysteine concentrations. In tumor-bearing rats, rectal epithelial proliferation was significantly correlated with the multiplicity of colorectal tumors and adenocarcinomas. These data collectively support our *a priori* hypothesis that the tumor-promoting effect of folic acid supplementation is in part mediated by increased cellular proliferation, which is considered to be a prerequisite for neoplastic transformation (57,58).

To date, there is no unequivocal evidence in humans demonstrating that folic acid supplementation promotes the progression of colorectal preneoplastic lesions. Studying the effect of folic acid supplementation on unresected colorectal polyps in humans is unethical. However, there exist a few clinical observations that support the potential tumor-promoting effect of folic acid supplementation on existing preneoplastic lesions. In the 1940s, the administration of folic acid was shown to accelerate the progression of leukemia in children (59). Recent epidemiologic studies have suggested that high intake and plasma levels of folate, largely from folic acid supplementation, are associated with an increased risk of colon (8) and breast (60,61) cancers. In the Aspirin/Folate Polyp Prevention Study (9), there was no effect of folic acid supplementation (1 mg/day) on the recurrence of adenomas at 6 years [relative risk 1.13; 95% confidence interval (CI) 0.93–1.37] in individuals with a history of colorectal adenomas. However, there was a 67% increased risk of advanced colorectal adenomas with a high malignant potential (relative risk 1.67; 95% CI = 1.00–2.80) and a >2-fold increased risk of multiple (≥3) colorectal adenomas (relative risk 2.32; 95% CI = 1.23–4.35) at 6 years (9). It is possible that folic acid supplementation might have promoted the progression of existing, undiagnosed preneoplastic lesions (e.g. ACF or microscopic adenomas) in these predisposed individuals (20,62). Another unexpected secondary finding from this trial was that folic acid supplementation was associated with a significant 2.6-fold increase in prostate cancer risk over 10 year follow-up among those subjects who agreed to follow-up and continued the assigned treatment after the completion of the trial (hazard ratio 2.58; 95% CI = 1.14–5.86) (10). Given the fact that the mean age of the study participants in this trial was 57 years (9,10), it is highly probable that some of these participants might have harbored precursor lesions in the prostate, which were allowed to progress more rapidly with folic acid supplementation. Two recent ecologic studies that examined a temporal postfortification trend of colorectal cancer incidence in the USA, Canada and Chile reported increased colorectal cancer rates in these countries (63,64) following fortification. These observation may reflect the accelerated development and growth of a preneoplastic lesion into a clinically detectable or symptomatic cancer. A recently published mathematical modeling reported that predicted colorectal cancer incidence rates under folic acid supplementation are mostly higher than rates without folic acid supplementation unless supplementation is initiated early in life (before age 20 years) (65). This modeling also indicated that the effect on colorectal cancer risk of starting folic acid supplementation late in life, though small, is usually detrimental (65).

In summary, notwithstanding the limitations associated with the AOM rat model of colorectal cancer and inherent differences in folate metabolism between humans and rats, our data suggest, for the first time, that folic acid supplementation promotes the progression of ACF. Our data therefore support the potential tumor-promoting effect of folic acid supplementation on existing preneoplastic colorectal lesions, a concern that was raised based on folate's biochemical function in one-carbon transfer reactions and on suggestive observations from previous preclinical (1,20) and human (9,10,59) studies. Dietary intake and blood measurements of folate in the USA and Canada have dramatically increased over the past decade due in part to mandatory folic acid fortification initiated in 1998 (21,22) and primarily to the up to 30–40% of the North American population who consume supplemental folic acid (23,66). Of particular concern is the fact that two analyses using the NHANES data found that postfortification, 32–38% of persons aged ≥60 years have high serum folate concentrations (>45.3 nmol/l) (21,22). Approximately, 25–50% of people by 50 years of age in the USA harbor asymptomatic colorectal adenomas, and the prevalence increases with age (67). This translates to 16–32 millions of Americans ≥50 years of age who might be susceptible to the tumor-promoting effect of folic acid supplementation. An even greater number of North Americans are probably to harbor ACF or microscopic adenomas in the colorectum and folic acid supplementation may accelerate the progression of these early precursors to colorectal adenomas and cancer. Definitive answers about the potential tumor-promoting effect of folic acid supplementation on preneoplastic lesions in the colorectum are beyond the reach of both observational epidemiologic studies and intervention trials in humans. However, our data from the present animal study, in conjunction with previous animal studies (18,19) and observations made in humans (9,10,59,63,64) suggest that there is sufficient cause for concern about the potentially deleterious effect of folic acid supplementation on colorectal cancer development and progression.

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