Effects of Dietary Folate on Intestinal Tumorigenesis in the Apc^{Min} Mouse[1]

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ABSTRACT

Dietary folate appears to be inversely related to colorectal cancer risk. This study investigated the effects of dietary intervention with folate on the development of intestinal polyps in Min ($Apc+/-$) mice. Weanling Min mice were fed diets containing 0, 2 (basal requirement), 8, or 20 mg folate/kg diet. At 3 and 6 months of dietary intervention, 50% of the mice from each group were sacrificed, and the small intestine and colon were analyzed for polyps and aberrant crypt foci (ACF). Serum folate concentrations accurately reflected dietary folate levels ($P < 0.001$). At 3 months, no significant difference in the average number of total small intestinal polyps was observed among the four groups. However, increasing dietary folate levels significantly reduced the number of ileal, but not duodenal or jejunal, polyps in a dose-dependent manner (P-trend $= 0.001$); folate supplementation at 20 mg/kg diet was associated with a 68–78% reduction in the number of ileal polyps compared with the other three diets ($P < 0.007$). The number of ileal polyps was inversely correlated with serum folate concentrations ($P = 0.03$). At 3 months, increasing dietary folate levels significantly decreased the number of colonic ACF in a dose-dependent manner ($P = 0.05$); the control and two folate supplemented diets significantly reduced the number of colonic ACF by 75–100% compared with the folate-deficient diet ($P < 0.04$). The number of colonic ACF was inversely correlated with serum folate concentrations ($P = 0.05$). No significant difference in the number of colonic adenomas was observed among the four groups at 3 months. At 6 months, no significant differences in the average number of total small intestinal, duodenal, and jejunal polyps, colonic adenomas, and colonic ACF were observed among the four groups. However, the folate-deficient diet had a 62–76% lower number of ileal polyps compared with the control and two folate-supplemented diets ($P < 0.003$). Serum folate concentrations, but not dietary folate levels, were directly correlated with the number of ileal polyps ($P = 0.006$). These data suggest that dietary folate supplementation suppresses the development of ileal polyps and colonic ACF in this model. However, at later time points, folate supplementation appears to have an opposite effect on ileal polyps. These data generally support the role of folate in intestinal tumorigenesis suggested in epidemiological studies and chemical carcinogen animal models. Notwithstanding the limitations associated with this model, these data suggest that the optimal timing and dose of folate intervention need to be determined for safe and effective folate chemoprevention.

INTRODUCTION

Dietary intake and blood measurements of folate appear to be inversely related to the risk of developing colorectal adenomas and cancer in the general population (1–15) and in subjects with chronic ulcerative colitis (16–18), a disease associated with an increased risk of both folate deficiency and colorectal dysplasia and cancer (19). Collectively, these studies suggest an ~40% reduction in the risk of colorectal neoplasia in individuals with the highest dietary intake and/or blood concentrations of folate compared with those with the lowest intake and/or blood concentrations (1–18). One of the most convincing lines of evidence comes from a recent prospective study involving 88,757 female nurses in the United States, which showed a 75% reduction in colorectal cancer risk in women using multivitamin supplements containing ≥400 μg of folic acid for ≥15 years after controlling for known confounding factors (15).

Two animal studies, conducted in the DMH[3] Sprague Dawley rat model of colorectal cancer, have supported a causal relationship between folate deficiency and colorectal cancer (20, 21). These studies have also shown a dose-dependent protective effect of modest levels of dietary folate supplementation up to four times the basal dietary requirement (20, 21). Levels of dietary folate greater than four times the dietary requirement did not convey further benefits; in fact, there was a nonsignificant trend toward increased colorectal tumorigenesis in rats fed a supraphysiological dose of folate ($20 \times$ the daily requirement; Ref. 21). These observations suggest that supplemental folate may have two distinct actions in this model. At modest levels of supplementation above the basal dietary requirement, folate appears to possess an inhibitory effect on the genesis and progression of colorectal neoplasms (20, 21). However, exceptionally high supplemental folate levels may promote the progression of chemically induced colorectal neoplastic foci (21). In support of this latter finding, dietary folate supplementation exceeding the basal requirement by 1000 times increased the development of aberrant crypt foci ACF, the probable earliest precursor of colorectal cancer (22), compared with a control diet in another study where azoxymethane (a metabolite of DMH) was used to induce colorectal cancer in Fischer 344 rats (23).

Although some similarities do exist, tumor development in chemical rodent models of colon cancer differs in several important respects from that observed in humans (24, 25). The chemically induced carcinomas often arise from flat foci of dysplasia rather than from adenomatous polyps. The relatively high doses of genotoxic chemical carcinogens differ from the natural etiological causes involved in most cases of sporadic colorectal cancer in humans. Most importantly, molecular genetics of chemical rodent models are significantly different from those observed in human colorectal carcinogenesis. It appears that the *Apc* and *p53* genes, two commonly mutated genes in human colorectal cancer (26), are either mutated to a much lesser extent or not mutated at all in these rodent models (27–31). Also, recent evidence suggests that chemically induced colorectal carcino-

[3] The abbreviations used are: DMH, dimethylhydrazine; ACF, aberrant crypt foci; *APC*, adenomatous polyposis coli; Min, multiple intestinal neoplasia ($Apc+/-$).

genesis in rodents involves alterations in unique colon cancer susceptibility genes that are not implicated in human colorectal cancer (32, 33).

Recently developed genetic murine models characterized by the spontaneous development of small intestinal and colonic tumors have provided an excellent opportunity to investigate the effects of environmental and genetic factors in both familial and sporadic colorectal carcinogenesis (25). One such model is Min mice, which carry a heterozygous germ-line mutation at codon 850 of the mouse *Apc* gene and develop approximately 25–75 small intestinal adenomas and 1–5 colorectal adenomas by 160–180 days, at which time they become moribund and die from anemia and intestinal obstruction (34, 35). The wild-type *Apc* allele is lost in adenomas from Min mice (36); and therefore, this model resembles the syndrome of familial adenomatous polyposis coli in humans (37). Min mice have been used to test the chemopreventive effect of aspirin and nonsteroidal anti-inflammatory drugs (38–43) and certain dietary factors (44–50).

This study investigated the effects of dietary intervention of folate on the development of small intestinal and colonic polyps and colonic ACF and to define the dose-response nature of folate chemoprevention in Min mice.

MATERIALS AND METHODS

This study was approved by the Animal Care Committee of the Samuel Lunenfeld Research Institute.

Mice. Min mice were bred at the Samuel Lunenfeld Research Institute on the C57BL/6J strain (original breeding pair from The Jackson Laboratory, Bar Harbor, ME). At our institution, Min mice develop ~30 small intestinal adenomas, <2 colonic ACF, and 1–5 colonic adenomas and have a life span of ~6 months.

Genotyping. Ear punch tissue was processed for genotyping using PCR-based assays to determine *Apc* status as described previously (51).

Dietary Intervention. Min mice were randomly assigned to receive an amino acid-defined diet (Dyets, Bethlehem, PA; Ref. 52) containing either 0, 2, 8, or 20 mg of folate/kg diet ($n = 19$–22 per group) from weaning at ~21 days of age. These diets constitute a standard method of inducing folate deficiency or providing supplemental dietary folate in rodents (52). The diet containing 0 mg folate/kg produces progressive folate deficiency of a moderate degree without anemia, growth retardation, or premature death through week 5, after which systemic folate indicators stabilize (20). Although this diet is completely devoid of folate, intestinal microflora are capable of *de novo* synthesis of folate, some of which is incorporated into tissue folate of the host; this prevents severe folate deficiency (53). Succinylsulfathiazole, which is conventionally used to create a severe folate deficiency (54), was not used in this study for the following reasons: (*a*) we wanted to avoid severe folate deficiency, which predictably causes severe growth retardation and premature death beyond 5–6 weeks (54, 55); (*b*) the consensus of epidemiological and clinical studies (1–18) and previous rat studies using DMH (20, 21) indicate that mild to moderate depletion of folate is sufficient to increase colorectal carcinogenesis. This folate-deficient diet is identical to that associated with an increased risk of colorectal neoplasms in our previous rat studies using DMH (20, 21). Two mg folate/kg diet is generally accepted as the basal dietary requirement for rodents (56). The diet containing 8 mg folate/kg represents folate supplement 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 rat studies (20, 21). Twenty mg of folate/kg diet (10 times the basal requirement) was chosen in this study because recent clinical studies demonstrated that folate supplementation in the amount of 12.5–25 times the daily requirement was able to modulate a biomarker of colorectal cancer in humans (57, 58). These diets contained 50 g of cellulose/kg, 60% calories as carbohydrates, 23% fat, and 17% L-amino acid (52). The amount of methyl donors, methionine, choline, and vitamin B_{12}, were 8.2 g, 2.0 g, and 50 μg per kg diet, respectively.

Specialized wire-bottomed stainless steel cages, which are used to minimize coprophagy when creating folate deficiency in rats (20, 21, 55), were not used in the present study because of ethical considerations. Diet and water were provided *ad libitum*. Body weights were recorded weekly.

Half of the mice in each group were sacrificed at 3 and 6 months after the beginning of dietary folate intervention (107–116 and 199–208 days of age) by cervical dislocation. These two time points of sacrifice were used to determine the effects of folate on small intestinal and colonic polyps and colonic ACF at early and late stages of intestinal tumorigenesis in this model, respectively. Most Min mouse chemoprevention studies have used 90–110 days of age as the time of sacrifice (38–50), and one study has also used >200 days of age as a late time point (43).

Enumeration of Small Intestinal Polyps and Colonic ACF and Adenomas. Intestines were immediately removed and flushed with Krebs buffer solution to remove fecal debris. The entire length of the small intestine and colon was opened longitudinally, laid flat on Whatman filter paper, and fixed for at least 3 h in 10% neutral buffered formalin. The mucosa of the fixed small intestine and colon was stained with methylene blue and examined in a blinded fashion for tumors and ACF by gross inspection and light microscopy as described previously (59). Previous studies have shown that all small intestinal polyps are adenomas (51). Representative small bowel and all colonic polyps were processed in a standard manner for H&E staining and histologically analyzed by a gastrointestinal pathologist blinded to the study groups.

Folate Concentration Determination. At the time of sacrifice, blood was withdrawn from the heart using a preheparinized 18-gauge needle into Vacutainer tubes containing EDTA and centrifuged at 800 × *g* for 10 min at 4°C. Serum was stored at −70°C in 0.5% ascorbic acid for the serum folate assay. Serum folate concentrations were measured by a microtiter plate assay using *Lactobacillus casei,* as described previously (60).

Statistics. The distribution of each variable was assessed graphically to determine whether it was normally distributed. For normally distributed variables, differences among the four diet groups were determined by one-way ANOVA. Variables that were not normally distributed were subjected to logarithmic transformation before performing one-way ANOVA. Fisher's least-significance-difference test was used for multiple comparisons. The test of linear trend was also performed to assess a trend in the number of small intestinal adenomas and colonic ACF as a function of dietary folate intake. The Pearson coefficient of regression model was used to assess correlation between variables. All significance tests were two sided and were considered statistically significant if the observed significance level was <0.05. Results are expressed as mean ± SE of the untransformed data. Statistical analyses were performed using SYSTAT 5 for Macintosh (Systat, Evanston, IL).

RESULTS

Body Weight. Growth curves were similar in the four groups; at no time point did the mean weights differ significantly among these groups. No premature death occurred. This finding indicates that folate deficiency in the mice fed 0 mg folate/kg diet was moderate; otherwise, growth retardation or premature death would have occurred (54).

Serum Folate Concentrations. At both 3 and 6 months after beginning of dietary intervention, the mean serum folate concentration of the mice fed 0 mg folate/kg diet was significantly lower than the control and two folate-supplemented groups ($P < 0.001$; Tables 1 and 2). At both time points, the mean serum folate concentrations increased incrementally as dietary folate levels were increased up to 8 mg/kg diet ($P < 0.03$; Tables 1 and 2). The mean serum folate concentration of the mice fed 20 mg folate/kg diet was not significantly different from those of the groups fed 2 and 8 mg folate/kg diet at both time points (Tables 1 and 2). Serum folate concentrations correlated directly with the levels of dietary folate ($r = 0.59$, $P < 0.001$ and $r = 0.54$, $P = 0.01$ at 3 and 6 months, respectively). The mean serum folate concentration of each group at 3 months was not significantly different from that of the corresponding group at 6 months, suggesting that serum folate concentrations reached a plateau by 3 months of dietary intervention.

The mean serum folate concentrations of the mice fed 0 mg fo-

Table 1 *Effects of dietary folate intervention on serum folate concentrations, small intestinal and colonic adenomas, and ACF at 3 months*[a]

Dietary folate (mg folate/kg diet)	0	2	8	20
No. of mice	10	11	10	11
Serum folate (ng/ml)	12.1 ± 3.9[i]	39.0 ± 3.4[ii]	56.0 ± 3.9[iii]	49.9 ± 3.5[ii, iii]
Average no. of small intestinal adenomas/mouse				
Duodenal adenomas	8.00 ± 1.7	6.54 ± 1.6	6.30 ± 1.7	8.36 ± 1.7
Jejunal adenomas	5.40 ± 2.3	9.36 ± 2.2	7.40 ± 2.3	6.18 ± 2.2
Ileal adenomas[b]	11.0 ± 1.6[i]	7.36 ± 1.6[i]	7.30 ± 1.6[i]	2.36 ± 1.6[ii]
Total adenomas	24.4 ± 4.4	23.3 ± 4.2	22.0 ± 4.4	16.9 ± 4.2
Average no. of colonic ACF/ mouse[c]	1.30 ± 0.3[i]	0.27 ± 0.3[ii]	0.20 ± 0.3[ii]	0.00 ± 0.3[ii]
Average no. of colonic adenomas/mouse	4.60 ± 0.7	4.18 ± 0.7	3.20 ± 0.70	4.18 ± 0.7

[a] Values are means ± SE. Means with different superscript letters in each row indicate statistical differences at *P* < 0.05 by Fisher's least-significance-difference multiple comparison test.
[b] *P*-linear trend = 0.001.
[c] *P*-linear trend = 0.05.

late/kg diet for 3 (12.1 ng/ml) and 6 (10.8 ng/ml) months were comparable with those observed in rats placed on the similar diet for 20 weeks in two previous experiments (9–23.9 ng/ml; Refs. 20 and 21). The mean serum folate concentrations of the mice fed the control (2 mg folate/kg) diet for 3 (39.0 ng/ml) and 6 (35.4 ng/ml) months were generally lower than that observed in rats placed on the similar diet for 20 weeks in a previous experiment (54.1 ng/ml; Ref. 21). The mean serum folate concentrations of the mice fed 8 mg folate/kg diet for 3 (56.0 ng/ml) and 6 (46.0 ng/ml) months were significantly lower than those observed in rats placed on the similar diet for 20 weeks in two previous experiments (89–98 ng/ml; Refs. 20 and 21). Also, in contrast to significantly elevated serum folate concentrations in rats fed 40 mg folate/kg diet compared with those fed 2 and 8 mg folate/kg diet in a previous experiment (144.6 ng/ml; Ref. 21), the mean serum folate concentrations of the mice placed on 20 mg folate/kg diet for 3 and 6 months were not significantly different from those of the mice fed 2 and 8 mg folate/kg diet. These observations suggest that folate absorption, metabolism, and excretion between rats and mice might be different.

Effects of Dietary Folate on Small Intestinal Polyps. All of the histologically analyzed representative small intestinal polyps (*n* = 5–10 per group) were confirmed to be adenomas in accordance with previous studies that have shown that all small intestinal polyps in Min mice are adenomas (51).

At 3 months, no significant difference in the average number of total small intestinal polyps was observed among the four groups (Table 1). When each of the segments of the small intestine was examined individually, however, increasing dietary folate levels significantly reduced the number of ileal, but not duodenal or jejunal, polyps (*P*-linear trend = 0.001; Table 1). Folate supplementation at 20 mg/kg diet was associated with a 68–78% reduction in the number of ileal polyps compared with the other three diets (*P* < 0.007; Table 1). Although the average number of ileal polyps was 34% higher in the mice fed 0 mg folate/kg diet than that of the mice placed on the control diet, this fell short of statistical significance (Table 1). No significant difference in the number of ileal polyps was observed between the control and 8 mg folate/kg groups (Table 1). When the number of ileal polyps was assessed as a function of actual serum folate concentrations, irrespective of dietary levels of folate, a highly significant inverse correlation between these two variables was demonstrated (*r* = −0.36, *P* = 0.031; Fig. 1*A*).

At 6 months, no significant differences in the average number of total small intestinal, duodenal, and jejunal polyps were observed among the four groups (Table 2). However, the mice fed 0 mg folate/kg diet had a 62–76% lower number of ileal polyps compared

with the control and two folate-supplemented groups (*P* < 0.003; Table 2). The average numbers of ileal polyps in the two folate-supplemented diets were not significantly different from that of the control group (Table 2). Serum folate concentrations, but not dietary folate levels, were directly correlated with the number of ileal polyps (*r* = 0.44, *P* = 0.006; Fig. 1*B*).

Effects of Dietary Folate on Colonic ACF and Adenomas. At 3 months, increasing dietary folate levels significantly decreased the number of colonic ACF (*P*-linear trend = 0.05; Table 1). The average numbers of colonic ACF in the control and two folate-supplemented groups were 75–100% lower than in the folate-deficient group (*P* < 0.04; Table 1). The average numbers of colonic ACF in the two folate-supplemented groups were not significantly different from that of the control group (Table 1). The number of colonic ACF was inversely correlated with serum folate concentrations (*r* = −0.32, *P* = 0.05; Fig. 1*C*). Min mice at our facility usually develop <2 colonic ACF. One mouse in the 0 mg folate/kg diet group, however, had 7 colonic ACF in the present study. When this outlier was excluded from the analysis, the average number of colonic ACF in the 0 mg folate/kg diet group was not significantly different from that of the control group (*P* = 0.097) but was still significantly higher than those in the two folate-supplemented groups (*P* < 0.05). Excluding this outlier strengthened the inverse association between the number of colonic ACF and dietary levels of folate (*P*-linear trend = 0.018), whereas it weakened the inverse association between the number of colonic ACF and serum folate concentrations (*r* = −0.062, *P* = 0.718).

All macroscopic colonic polyps were harvested and histologically analyzed. Only histologically confirmed colonic adenomas were included in subsequent analyses. No significant difference in the average number of colonic adenomas was observed among the four groups at 3 months (Table 1). No significant correlation between the number of colonic adenomas and serum folate concentrations was observed at 3 months.

At 6 months, no significant differences in the average number of colonic ACF and adenomas were observed among the four groups (Table 2). No significant correlations between the number of colonic ACF and adenomas and serum folate concentrations were observed.

DISCUSSION

The results from this study where dietary folate intervention was provided for 3 months demonstrate that dietary and serum levels of folate are inversely related to the number of ileal, but not total small intestinal, duodenal, and jejunal, polyps in Min mice. This observation generally corroborates findings from epidemiological studies (1–18)

Table 2 *Effects of dietary folate intervention on serum folate concentrations, small intestinal and colonic adenomas, and ACF at 6 months*[a]

Dietary folate (mg folate/kg diet)	0	2	8	20
No. of mice	9	11	9	8
Serum folate (ng/ml)	10.8 ± 3.2[i]	35.4 ± 2.9[ii]	46.0 ± 4.0[iii]	43.3 ± 3.4[ii, iii]
Average no. of small intestinal adenomas/mouse				
Duodenal adenomas	12.70 ± 3.2	9.73 ± 2.8	9.89 ± 3.2	5.62 ± 3.3
Jejunal adenomas	3.56 ± 3.1	9.46 ± 2.8	8.22 ± 3.1	7.88 ± 3.3
Ileal adenomas	1.67 ± 1.6[i]	7.09 ± 1.6[ii]	5.33 ± 1.8[ii]	4.38 ± 2.0[ii]
Total adenomas	17.9 ± 6.5	26.3 ± 5.9	23.4 ± 6.5	17.9 ± 6.9
Average no. of colonic ACF/ mouse	0.33 ± 0.3	0.27 ± 0.2	0.56 ± 0.3	0.38 ± 0.3
Average no. of colonic adenomas/mouse	2.56 ± 0.8	2.82 ± 0.7	2.00 ± 0.8	4.43 ± 0.9

[a] Values are means ± SE. Means with different superscript letters in each row indicate statistical differences at *P* < 0.003 by Fisher's least-significance-difference multiple comparison test.
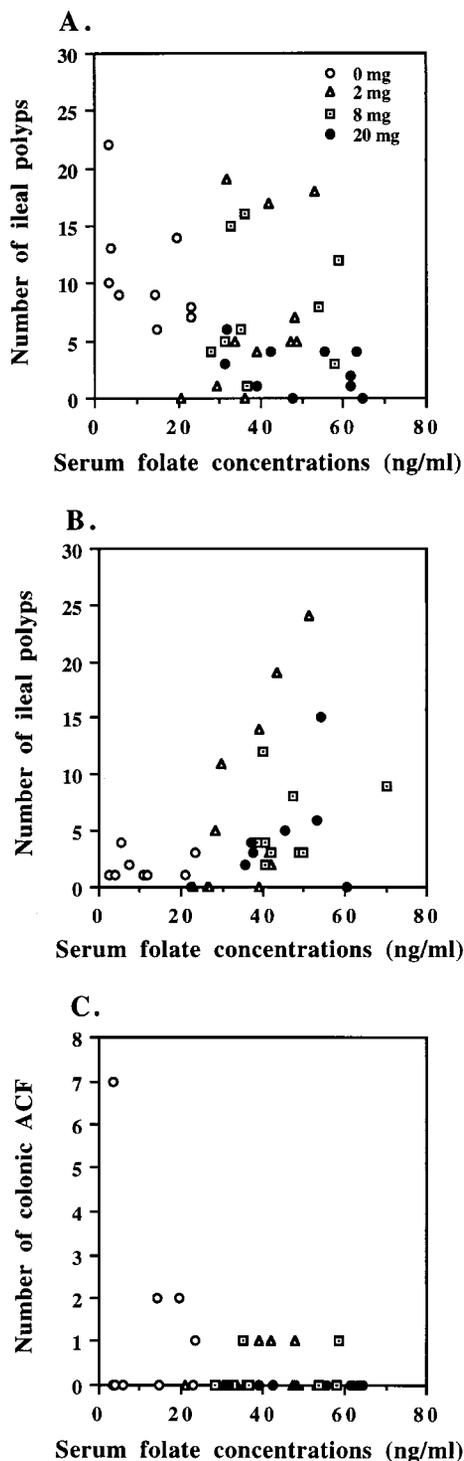
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A.

B.

C.

Fig. 1. *A*, correlation between the number of ileal polyps and serum folate concentrations at 3 months. A significant inverse correlation between these variables was observed ($r = -0.36$, $P = 0.031$). *B*, correlation between the number of ileal polyps and serum folate concentrations at 6 months. A significant direct correlation between these variables was observed ($r = 0.44$, $P = 0.006$). *C*, correlation between the number of colonic ACF and serum folate concentrations at 3 months. A significant inverse correlation between these variables was observed ($r = -0.32$, $P = 0.05$).

that have suggested that a mild-to-moderate reduction of folate status is sufficient to enhance colorectal cancer risk, and that modest increases in dietary folate intake above the required daily amount and in blood levels of folate are associated with decreased colorectal cancer risk in a dose-dependent fashion (1–18). The present data also support findings from two animal studies conducted in the DMH rat model of

colorectal cancer (20, 21). However, although the present study suggests that folate supplementation at 10 times the basal requirement is the most effective chemopreventive dietary level of folate in Min mice, prior animal studies suggested that the most effective chemopreventive dose of folate in rats is 4 times the basal requirement, beyond which no further benefits were observed (20, 21). These differences are most likely related to species differences and methods of tumor induction.

In contrast to the observations made at 3 months, at 6 months of dietary folate intervention, serum, but not dietary, levels of folate are directly associated with the number of ileal polyps in Min mice. Dietary folate intervention had no significant effects on the number of total small intestinal, duodenal, and jejunal polyps at 6 months. The mice fed 0 mg folate/kg diet had a 62–76% lower number of ileal polyps compared with the control and two folate-supplemented groups, whereas no significant difference was observed among the control and two folate-supplemented groups (Table 2). This observation suggests that folate depletion might have caused regression of established ileal polyps in Min mice when dietary folate intervention was provided for 6 months. However, it is possible that the presence of a large number of intestinal tumors might have substantially altered the response to dietary folate in Min mice, either by simply interfering with feeding or by more complex effects on metabolism known to occur in tumor-bearing patients and animals, and that this might have been responsible for the data observed at 6 months. In the present study, no conclusion can be drawn regarding whether folate supplementation had a promoting effect on the progression of established ileal polyps. It appears that the number of small intestinal polyps has reached maximum by 3 months in Min mice, as evidenced by no significant difference in the number of small intestinal polyps in the control group at 3 and 6 months of dietary folate intervention (Tables 1 and 2). Therefore, a possible additional promoting effect of folate supplementation on ileal polyps beyond the expected maximum number of polyps observed in Min mice on the control diet would have not been observed.

The inhibitory effect of folate depletion on established ileal polyps observed at 6 months is consistent with the biochemical function of folate. Folate plays an important role in DNA synthesis and replication (61). Consequently, folate deficiency in tissues with rapidly replicating cells results in ineffective DNA synthesis. In neoplastic cells where DNA replication and cell division are occurring at an accelerated rate, interruption of folate metabolism causes ineffective DNA synthesis, resulting in inhibition of tumor growth (62). This has been the basis for antitumor therapy using a number of antifolate agents, including methotrexate and 5-fluorouracil (62). Experimentally, it has been shown that growth of a transplanted cancer is inhibited in folate-deficient rats (63), that folate deprivation reduces the growth of virally induced cancers (64), and that the time required for developing nerve sheath tumors in transgenic mice is significantly delayed by restricting the level of folate in diet (65). Furthermore, the addition of folate to established tumors has been shown to cause an "acceleration phenomenon." For instance, children with acute leukemia treated with folate supplementation experienced an accelerated progression of leukemia (66). Taken together, these observations suggest that folate deficiency has an inhibitory effect on progression of established neoplasms or may even cause regression of established tumors.

At 3 months of dietary folate intervention, dietary and serum levels of folate were inversely correlated with the number of colonic ACF in Min mice. The control and two folate-supplemented groups had significantly lower numbers of colonic ACF than the folate-deficient diet group. These observations were slightly weakened when one extreme outlier was excluded from the analysis. In contrast, no sig-

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nificant difference in the average number of colonic adenomas was observed among the four groups, and no significant correlation between the number of colonic adenomas and serum folate concentrations was observed. The data on ACF suggest that moderate folate deficiency enhances, whereas folate sufficiency and supplementation suppress, the initiation phase of colorectal tumorigenesis in Min mice. In contrast, the data on colorectal adenomas suggest that folate status does not play a major role in the progression of established colorectal ACF to adenomas. These observations in Min mice are different from findings from prior animal studies that showed protective effects of dietary folate supplementation both on the development and progression of colonic neoplasms in rats (20, 21). It should be noted that the relationship between ACF and colonic adenomas in Min mice is not entirely straightforward. Although the ACF is generally regarded as the earliest precursor of colorectal adenomas and cancer in humans and in chemical carcinogen models (22), ACF in Min mice do not appear to contribute significantly to the colon adenoma population (51). This is supported by the observation that ACF develop postnatally, whereas colon adenomas arise perinatally in Min mice (51). The Min mouse develops very few ACF (<2) compared with chemical rodent models or humans at risk of developing colorectal cancer (22). Therefore, the protective effect of folate on ACF observed in the present study needs to be confirmed in chemical rodent models and other *Apc* knockout models where more number of ACF are observed to develop.

In contrast to its inhibitory effect on ileal polyps, dietary folate deficiency had no significant effect on the number of ACF and adenomas in the colon at 6 months. Also, no significant correlations between the number of colonic ACF and adenomas and serum folate concentrations were observed at 6 months. It is possible that rapidly developing ileal polyps in Min mice are most susceptible to the effect of folate depletion, whereas colonic ACF and adenomas with a normal or slower growth rate may not. Another possibility is a lack of time for folate deficiency to exert its effect on established colonic neoplastic foci because most mice die by 6 months of age.

The effects of dietary folate appear to be site specific in the small intestine at both 3 and 6 months. In the present study, the most susceptible site to the dietary manipulation by folate was the ileum. This observation is consistent with findings from previous studies that have shown that the distal small intestine is most susceptible to the actions of chemopreventive agents or dietary factors in Min mice (40, 43, 49). The lack of effect of dietary folate on colorectal adenomas in the present study is also consistent with previous studies that have demonstrated that the colon is relatively resistant to the action of chemopreventive agents or dietary factors in Min mice (38, 41, 42, 47, 48, 50).

The degree of folate depletion observed in the mice fed 0 mg folate/kg diet was moderate, as evidenced by the serum folate concentrations as well as the absence of growth retardation and premature death. Although coprophagy was not specifically prevented using special cages in the present study because of ethical considerations, the degree of folate depletion in mice fed 0 mg folate/kg diet at both 3 and 6 months (serum folate concentrations 12.1 and 10.8 ng/ml, respectively) was comparable with that observed in prior experiments, where rats were placed on the similar diet and in special cages to minimize coprophagy for 20 weeks (9–23.9 ng/ml; Refs. 20, 21). However, there were several differences between Min mice and rats with regard to serum folate concentrations: (*a*) the mean serum folate concentrations of the mice fed 0 and 8 mg folate/kg diet for 3 and 6 months were lower than the corresponding levels observed in rats placed on the similar diets for 20 weeks in two previous experiments (20, 21); (*b*) in contrast to significantly elevated serum folate concentrations in rats fed 40 mg folate/kg diet compared with those fed 2 and

8 mg folate/kg diet in a previous experiment (21), the mean serum folate concentrations of the mice placed on 20 mg folate/kg diet for 3 and 6 months were not significantly different from those of the mice fed 2 and 8 mg folate/kg diet. These observations suggest that folate absorption, metabolism, and excretion between rats and mice might be different.

Although serum folate concentrations were directly correlated with the levels of dietary folate ($r = 0.54-0.59$, $P < 0.01$) at both time points, a wide range of serum folate concentrations was observed within each dietary group. This is probably related to variable food intake and different folate absorption, metabolism, and excretion of individual mice within each dietary group. Thus, actual serum folate concentrations rather than dietary levels of folate might be a better predictor of the chemopreventive effect of folate on intestinal tumorigenesis in Min mice. Another notable observation in the present study is that the effect of the diets containing 2 and 8 mg folate/kg on ileal polyps and colonic ACF was not significantly different, whereas the two extreme diets containing 0 and 20 mg folate/kg demonstrated effects. The data from the present study suggest that, at least in Min mice, dietary folate supplementation at 10 times the basal requirement (*i.e.*, 20 mg folate/kg diet) is the most effective chemopreventive dose. Taking data from prior rat studies (20, 21, 23) and the present study, it appears that folate supplementation ranging from 4 times to 10 times is effective for the chemoprevention of colorectal and small intestinal tumorigenesis in rodents. However, the optimal dose for safe and effective folate chemoprevention in humans still needs to be established.

The present study did not investigate the mechanisms by which folate supplementation can suppress intestinal tumorigenesis at 3 months in Min mice. The mechanisms by which dietary folate can modulate colorectal carcinogenesis have not been clearly elucidated (67, 68). The sole biochemical function known for folate is mediating the transfer of one-carbon moieties (61). In this role, folate is an important factor in DNA synthesis, stability and integrity, repair and methylation, aberrations of which are implicated in carcinogenesis (67, 68). A growing body of *in vivo* and *in vitro* evidence suggests that folate deficiency is associated with DNA damage, impaired DNA repair, abnormal DNA methylation, and increased mutagenesis, which can be overcome by folate supplementation (67, 68).

Several limitations of the Apc^{Min} murine model need to be acknowledged: (*a*) the predominant phenotype in this model is the development of small intestinal polyps in contrast to colon polyps in humans; (*b*) Apc^{Min} mice do not develop small intestinal or colonic adenocarcinomas because they become moribund as a consequence of florid polyposis; (*c*) as described previously, the contribution of ACF to the adenoma population in this model is not clearly established; (*d*) this model may reflect only inherited types of accelerated tumorigenesis, such as familial adenomatous polyposis and not sporadic colorectal carcinogenesis; and (*e*) the distal small intestine is most susceptible, whereas the colon is relatively resistant, to chemopreventive actions of drugs and nutritional factors in this model. Because of this site-specific susceptibility of chemoprevention, the extrapolation of information concerning the effects of chemopreventive agents in this model to humans is therefore uncertain. Nonetheless, the Apc^{Min} mouse appears to be an excellent model to study chemopreventive effects of dietary factors and drugs on intestinal tumorigenesis because of the spontaneous development of small intestinal and colonic polyps, genetic similarities to human colorectal cancer, and the accelerated nature of tumorigenesis, which provides an opportunity to determine effects of chemopreventive agents on tumorigenesis in a relatively short time. Furthermore, important information concerning mechanisms of inhibition of tumor development can be generated in this model.

In summary, our data suggest that dietary folate supplementation suppresses the development of ileal polyps and colonic ACF in the Min mouse. This observation corroborates prior epidemiological observations (1–18) and data from chemical carcinogen rodent models (20, 21) and renders further support for the possible chemopreventive role of folate supplementation in colorectal carcinogenesis. The present study also suggests that folate status might have an opposite effect on established ileal polyps. Notwithstanding the limitations associated with animal models, data from the present and prior animal studies (20, 21) suggest that folate chemoprevention of colorectal cancer in humans merits further consideration, but the optimal timing and dose of folate intervention need to be established for safe and effective chemoprevention in humans.

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