Short Communication

Effects of Dietary Folate on Ulcerative Colitis-Associated Colorectal Carcinogenesis in the Interleukin 2- and β2-Microglobulin-deficient Mice

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Abstract
Folate supplementation may reduce the risk of colorectal dysplasia and cancer in subjects with chronic ulcerative colitis (UC). The interleukin (IL) 2- and β2-microglobulin (β2m)-deficient (IL-2null × β2mnull) mice spontaneously develop colon cancer in the setting of chronic UC. This study investigated the effects of dietary folate on the development of UC-associated colon cancer in the IL-2null × β2mnull mice. Weaning IL-2null × β2mnull mice were randomized to receive 0 (deficient; n = 40), 2 (basal requirement; control; n = 46), or 8 (supplemented; n = 36) mg folate/kg diet for 32 weeks. At necropsy, all carcasses were examined and histologically classified as dysplasia or adenocarcinoma. The incidence of high-grade lesions (high-grade dysplasia/carcinoma in situ and invasive adenocarcinoma) in the folate-supplemented group was 46% lower than that in the control group (35.3% versus 65.1%, P = 0.009). The incidence of high-grade lesions in the folate-deficient group was also 49% lower than that in the control group (33.3% versus 65.1%, P = 0.007). The higher mortality rate in the folate-deficient group compared with the other two groups (28% versus 6.5% and 5.6%, P < 0.02) partially accounted for the low incidence of high-grade lesions in this group. These data indicate that dietary folate supplementation at 4× the basal dietary requirement significantly suppresses UC-associated colorectal carcinogenesis in the IL-2null × β2mnull mice. These data also suggest that folate deficiency may inhibit colorectal carcinogenesis in chronic UC. However, the high mortality observed in the folate-deficient group precludes a definitive conclusion concerning the effect of folate deficiency on UC-associated colorectal carcinogenesis in this model.

Introduction
Chronic UC is associated with a 10- to 40-fold increased risk of developing CRC compared with the general population (1). A recent meta-analysis of all published studies reporting a CRC risk in UC since 1925 has reported the risk for any patients with UC to be 2% at 10 years, 8% at 20 years, and 18% after 30 years of disease (2). Two strategies are currently used to prevent CRC in patients with chronic UC: (a) colonoscopic surveillance; and (b) prophylactic colectomy (1, 3). These strategies are, however, associated with inherent limitations as well as significant morbidity and even mortality (1). Another strategy that has not yet been studied extensively is the prevention of UC-associated CRC by nutritional factors and chemopreventive agents. In this regard, studies have suggested that folate (4–6), short-chain fatty acids (7), ursocholic acid (8), and 5-amino-5-deoxyadenosine (9) may reduce the risk of UC-associated CRC.

Dietary intake and blood levels of folate, a water-soluble B vitamin, have been shown to be inversely associated with the risk of developing sporadic CRC (10, 11). Collectively, these studies suggest an approximately 40% reduction in the risk of CRC in individuals with the highest dietary intake of folate compared with those with the lowest intake (10, 11). Although animal studies are generally supportive of a causal relationship between folate deficiency and CRC risk (12–16), some animal studies have shown that the dose and timing of folate intervention are critical in providing safe and effective chemoprevention; exceptionally high supplemental levels of folate (13, 17, 18) and folate intervention provided after microscopic neoplastic foci are established in the colorectal mucosa (14, 15) promote, rather than suppress, colorectal carcinogenesis.

Folate may also play a role in UC-associated colorectal carcinogenesis. Lashner et al. (4) reported that individuals with long-standing UC taking folate supplementation had a nonsignificant 62% lower incidence of colorectal dysplasia and cancer compared with those not receiving folate supplementation (OR, 0.38; 95% CI, 0.12–1.20). In another study (5), the risk of colorectal dysplasia and cancer was found to be significantly decreased by 18% for each 10 ng/ml increase in RBC folate concentrations in patients with UC (OR, 0.82; 95% CI, 0.68–0.99). In a recent study (6), folate supplementation was inversely related to the risk of colorectal neoplasia in subjects with long-standing UC in a dose-dependent manner (relative...
risk, 0.54 and 0.76 for 1.0 and 0.4 mg folate/day, respectively). Although these studies included small sample sizes and were associated with inherent limitations associated with retrospective study designs, these studies nevertheless suggest that the purported inverse relationship between folate status and the risk of CRC in chronic UC merits further consideration. Although megaloblastic anemia is rare, patients with UC often demonstrate depressed blood concentrations of folate due to the frequent use of sulfasalazine, a known folate antagonist (19), inadequate nutritional intake, and intestinal losses from inflammation (20).

A recently developed and characterized genetically predisposed murine model (the IL-2null × βmnull mice) of chronic UC with the spontaneous development of CRC provides an excellent opportunity to investigate the potential chemopreventive effect of folate on UC-associated CRC because the clinical features and molecular genetics are similar to those of UC-associated CRC in humans. The IL-2null × βmnull mice were generated by crossing mice deficient in CD8+ T cells and MHC class I expression, as a result of a targeted mutation in the βm gene, with mice deficient in IL-2 (21). The IL-2null × βmnull mice develop mild to moderate colitis with diarrhea and mild wasting, and some develop rectal prolapse, usually between 8 and 12 weeks, and most mice recover with normal stools, weight gain, and normal appearance and survive beyond 6 months, suggesting active disease followed by remission from colitis (21). Histologically, 75% of these mice have mild to moderate colonic inflammation restricted to the mucosa involving the entire colon, and 25% have no inflammation at the time of necropsy (21). Some of the IL-2null × βmnull mice develop adenocarcinoma in the proximal half of the colon between 6 and 12 months (22). No tumors have been observed in mice < 6 months of age, suggesting that adenocarcinomas arise only after a prolonged period of colonic inflammation (22). All of the tumors are well to moderately differentiated adenocarcinomas invading into or through the muscularis propria (22). The nature and frequency of p53 mutations and mismatch repair defects in CRC arising in IL-2null × βmnull mice are similar to those of human sporadic and UC-associated CRC, whereas the mutational spectrum of the Apc gene is slightly different (23). This study investigated the effects of dietary folate on the development of UC-associated colorectal carcinogenesis in the IL-2null × βmnull mice.

Materials and Methods

Animals. This study was approved by the Animal Care Committee of the University of Toronto. The generation of the IL-2null × βmnull mice on a C57BL/6 × 129/OLA or 129/Sv mixed background has been described previously (21, 22). The IL-2null × βmnull mice were bred at the University of Toronto from original breeding pairs provided by Dr. C. Terhorst (Division of Immunology, Beth Israel Deaconess Medical Center, Harvard Medical School). Mouse tail tip was processed for genotyping using a PCR-based assay to determine IL-2 status as described previously (15, 25). Diet and water were provided ad libitum. Body weights were recorded weekly. All of the mice were monitored daily for clinical evidence of illness or morbidity. Given the average timing of the development of CRC in this model (between 6 and 8 months; Ref. 22), all of the mice from each group were sacrificed at 32 weeks after the beginning of dietary folate intervention (or 35 weeks of age) by cervical dislocation.

Enumeration of Colorectal Neoplasms and Colitis Score. At necropsy, the colorectum was removed and flushed with Krebs buffer solution to remove fecal debris. The entire length of the colorectum was opened longitudinally, laid flat on Whatman filter paper, and fixed in 10% neutral buffered formalin. All macroscopic tumors were identified and harvested under a dissecting microscope, processed in a standard manner for H&E staining, and histologically classified as low-grade dysplasia, high-grade dysplasia/carcinoma in situ, or invasive adenocarcinoma according to the previously described criteria (28) by a gastrointestinal pathologist (A. M.) blinded to the study group. In addition, the severity of colitis was graded by the same pathologist in a blinded fashion based on histological examination of the distal colon (within 1 cm from the rectum) as follows: grade 0, normal histological findings; grade 1, mild thickening of the colon with often focal involvement of the mucosa; grade 2, moderate thickening of the colon with diffuse involvement of the mucosa; or grade 3, marked thickening of the colon with occasional crypt abscesses and the presence of focal lymphoid aggregates (29).

Serum Folate Concentration Determination. Blood was withdrawn from the tail of each mouse at 8, 16, and 24 weeks and from the heart at 32 weeks (at necropsy) after the beginning of dietary intervention and processed for the determination of serum folate concentrations by a microtiter plate assay using Lactobacillus casei as described previously (30).

Statistics. The distribution of each variable was assessed graphically to determine whether it was normally distributed. For normally distributed variables, differences among the three diet groups were determined by the Kruskal-Wallis non-parametric ANOVA. The Mann-Whitney test was used for pairwise group comparisons. For categorical response variables, differences among the three groups were assessed by Pearson χ² test. The test of linear trend was also performed to assess a trend in changes in values over the study period. All signifi-
cancer tests were two sided and considered statistically significant if the observed significance level was <0.05. Results are expressed as mean ± SE. Statistical analyses were performed using SPSS (version 10).

Results

Body Weight, Clinical Features, and Mortality Rate. The mean weights were not significantly different among the three dietary groups until week 13 of dietary intervention. However, between weeks 14 and 27 of dietary intervention, the mean weight of the folate-deficient mice was 9–18% lower than that of the folate-supplemented mice (P < 0.05). From week 28 until the time of necropsy, there was no significant difference in weights between these two groups. No significant difference in weights was observed between the folate-deficient and control mice and between the control and folate-supplemented mice between week 14 and the time of necropsy. These observations do contrast with previous studies that observed no significant difference in body weights between rodents fed the same folate-deficient diet and those fed the same control and folate-supplemented diets for 24–25 weeks (13, 14, 31).

Most of the IL-2 null × β2mnull mice in the three dietary groups showed signs of diarrhea, and several developed rectal prolapse and appeared to be ill with mild wasting between 8 and 12 weeks of age. However, most of these mice recovered with normal stools and weight gain. Twenty-five percent (10 of 40) of the mice receiving the folate-deficient diet died during the study period as compared with 6.5% (3 of 46) of the mice receiving the folate-supplemented diet (P < 0.02). These mice were found dead without warning symptoms and signs, and the exact cause(s) of death could not be determined. There was no evidence for the primary involvement of a contagious pathogen as determined by histological and microbiological analyses. Furthermore, serological tests for antibodies against the most common murine pathogens were negative in sentinel mice. These prematurely dead mice were excluded from further analyses pertaining to colitis score and tumor incidence.

Serum Folate Concentrations. The mean serum folate concentrations during the study were significantly different among the three dietary groups (P < 0.001; Fig. 1). The mean serum folate concentrations progressively decreased during the study in the folate-deficient group (P < 0.01, linear trend), whereas those of the control and folate-supplemented groups did not significantly change over time (Fig. 1). The mean serum folate concentrations of the three dietary groups were comparable with those observed in mice placed on the corresponding diets for 24 weeks in a previous experiment (14).

Colitis Score. Dietary folate intervention had no significant effects on the severity of colitis of the distal colon. As shown in Table 1, the distribution of colitis severity and the mean colitis score were not significantly different among the three groups (P = 0.94 and P = 0.93, respectively). Sixty-eight percent of the mice had colonic inflammation with the following histological index of severity: grade 1, 26%; grade 2, 32%; and grade 3, 10%. Thirty-two percent of the mice had no histological evidence of colitis. This finding is consistent with the previous observation made in this model (22) and indicates that the severity of colitis in these mice is mild to moderate.

Effects of Dietary Folate on Colonic Dysplasia and Cancer. Consistent with the previous observation (22), all colonic dysplasias and cancers were located in the proximal colon in the present study. The proportion of colonic dysplasia and cancer was significantly different among the three dietary groups mainly due to the difference in distribution of high-grade dysplasia/carcinoma in situ and invasive adenocarcinoma (i.e., high-grade lesions; Table 2; P = 0.035). The incidence of high-grade lesions in the folate-supplemented group was 46% lower than that of the control group (35.3% versus 65.1%, P = 0.009; Table 2). The incidence of high-grade lesions in the folate-deficient group was also 49% lower than that of the control group (33.3% versus 65.1%, P = 0.007; Table 2). No significant difference in the incidence of high-grade lesions was observed between the folate-deficient and folate-supplemented groups (P = 0.87; Table 2).

Because a significantly higher proportion of the folate-deficient mice had died before they could develop high-grade lesions, we examined the combined end point of death or high-grade lesions. The incidence of this combined end point in

**Table 1.** Effect of dietary folate deficiency and supplementation on the severity of colitis in the distal colon.

<table>
<thead>
<tr>
<th>Colitis score</th>
<th>Dietary levels of folate (mg/kg diet)</th>
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<tbody>
<tr>
<td></td>
<td>0 (n = 30)</td>
</tr>
<tr>
<td></td>
<td>2 (n = 43)</td>
</tr>
<tr>
<td></td>
<td>8 (n = 34)</td>
</tr>
<tr>
<td>0</td>
<td>8 (26.7%) 16 (37.2%) 10 (29.4%)</td>
</tr>
<tr>
<td>1</td>
<td>9 (30.0%) 9 (29.9%) 10 (29.4%)</td>
</tr>
<tr>
<td>2</td>
<td>10 (33.3%) 14 (32.6%) 10 (29.4%)</td>
</tr>
<tr>
<td>3</td>
<td>3 (10.0%) 4 (9.3%) 4 (11.8%)</td>
</tr>
</tbody>
</table>

The distribution of colitis severity and the mean colitis score in the distal colon were not significantly different among the three dietary groups (P = 0.94 and P = 0.93, respectively), suggesting that dietary folate levels had no significant effect on the severity of colitis. Colitis score: grade 0, normal histological findings; grade 1, mild thickening of the colon with often focal involvement of the mucosa; grade 2, moderate thickening of the colon with diffuse involvement of the mucosa; grade 4, marked thickening of the colon with occasional crypt abscesses and the presence of focal lymphoid aggregates (29).

![Fig. 1.](image-url) Mean serum folate concentrations in three groups of mice fed different levels of dietary folate for 32 weeks were significantly different during the study period. Different letters at each time point indicate statistically significant differences (P < 0.001). The mean serum folate concentrations progressively decreased during the study period in the folate-deficient group (P < 0.01, linear trend), whereas those of the control and folate-supplemented groups did not significantly change over time. Values are expressed as mean ± SE.
The distribution of colonic dysplasia and cancer was significantly different among the three dietary groups due to the difference in the distribution of high-grade lesions (high-grade dysplasia/carcinoma in situ and invasive adenocarcinoma, \( P = 0.035 \), Pearson \( \chi^2 \) test). The incidence of high-grade lesions was significantly lower in the folate-deficient (33.3%) and folate-supplemented (35.3%) groups than in the control (2 mg folate/kg diet; 65.1%) group (\( P < 0.01 \)), whereas no significant difference was observed between the folate-deficient and folate-supplemented groups (\( P = 0.87 \)).

The proportion of high-grade lesions was not significantly different among the different grades of colitis (\( P = 0.27 \); Table 3), suggesting that the severity of colitis did not affect the development of high-grade lesions in the present study. Up to 50% of the mice without histological evidence of colitis had high-grade lesions (Table 3).

The multiplicity of high-grade lesions (defined as the number of high-grade lesions/lesion-bearing mouse) was significantly different among the three dietary groups (\( P = 0.011 \)). The mean number of high-grade lesions in the folate-deficient group was significantly lower than that in the control group (0.88 ± 0.25 versus 1.91 ± 0.27; \( P = 0.009 \)). The mean number of high-grade lesions in the folate-supplemented group was also significantly lower than that in the control group (1.16 ± 0.38 versus 1.91 ± 0.27; \( P = 0.022 \)). No significant difference in the multiplicity of high-grade lesions was observed between the folate-deficient and folate-supplemented groups.

Discussion

The results from the present study demonstrate that dietary folate supplementation at 4× the basal dietary requirement significantly suppresses colorectal carcinogenesis in the IL-2(m−/−)× β_m null mouse model of UC-associated CRC. Given that the significant inhibitory effect of folate supplementation was observed on the incidence of high-grade lesions, and not on earlier stages of carcinogenesis, as compared with the control diet, dietary folate supplementation likely exerted its antiproliferative effect on the progression of neoplastic foci to high-grade lesions. The inhibitory effect of dietary folate supplementation on colorectal carcinogenesis appears to be independent of the severity of colonic inflammation. These observations corroborate findings from epidemiological studies that have suggested that folate supplementation (0.4–1.0 mg/day) may reduce the risk of colorectal dysplasia and cancer in subjects with chronic UC (4–6). Furthermore, these data are consistent with the purported chemopreventive effect of folate on sporadic colorectal carcinogenesis observed in epidemiological (10, 11) and animal (12–16) studies.

One unexpected finding from the present study is that dietary folate depletion of a mild degree also significantly reduced the incidence of high-grade lesions in the IL-2(m−/−)× β_m null mice compared with the control diet. One explanation for this observation is related to the unexpected high mortality in the folate-deficient mice. In contrast to previous studies in which the same folate-deficient diet was not associated with any premature death in IL-2(m−/−)× β_m competent rodents for up to 25 weeks (13, 14, 31), 25% of the folate-deficient IL-2(m−/−)× β_m null mice died of undetermined cause(s) compared with 5.6–6.5% in the control and folate-supplemented mice in the

### Table 2

<table>
<thead>
<tr>
<th>Dietary levels of folate (mg/kg diet)</th>
<th>Incidence of high-grade lesions (%)</th>
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<tbody>
<tr>
<td>0 (n = 30)</td>
<td>13 (43.3%)</td>
</tr>
<tr>
<td>2 (n = 43)</td>
<td>11 (25.6%)</td>
</tr>
<tr>
<td>8 (n = 34)</td>
<td>15 (44.1%)</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Colitis score</th>
<th>Presence of high-grade lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>0 (n = 34)</td>
<td>16 (47.0%)</td>
</tr>
<tr>
<td>1 (n = 28)</td>
<td>10 (35.7%)</td>
</tr>
<tr>
<td>2 (n = 34)</td>
<td>19 (55.9%)</td>
</tr>
<tr>
<td>3 (n = 11)</td>
<td>5 (45.5%)</td>
</tr>
</tbody>
</table>

*The proportion of high-grade lesions was not significantly different among the different grades of colitis (\( P = 0.27 \), Pearson \( \chi^2 \) test), suggesting that the severity of colitis did not affect the development of high-grade lesions.
present study. Our analysis determining the effect of dietary folate on the combined end point of death or high-grade lesions suggests that the high mortality in the folate-deficient group partially explains the observed lower incidence of high-grade lesions in this group compared with the control group. Another explanation for this observation relates to the dual modulatory effect of folate status on carcinogenesis depending on the timing of folate intervention. Animal studies have clearly demonstrated that folate deficiency enhances, whereas folate supplementation suppresses, neoplastic transformation in normal colorectal epithelium (14, 15). By contrast, folate deficiency inhibits the progression and may even cause regression of established colorectal neoplastic cells, whereas folate supplementation promotes the progression of pre-existing colorectal neoplastic foci (14, 15). This dual effect of folate on carcinogenesis depending on the timing of intervention has also been demonstrated in other in vitro and in vivo models (32, 33). Interruption of folate metabolism in rapidly replicating neoplastic cells to cause ineffective DNA synthesis and hence the inhibition of tumor growth has been the basis for antitumor therapy using antifolate agents (34). Therefore, it is possible that dietary folate deprivation might have inhibited the progression of, or caused regression, of neoplastic foci in the colorectal mucosa in the IL-2null mice.

The present study did not investigate the mechanisms by which folate supplementation can suppress colorectal carcinogenesis in the IL-2null × βmnull murine model of UC-associated CRC. The mechanisms by which dietary folate can modulate colorectal carcinogenesis have not been clearly elucidated (10, 11). The sole biochemical function known for folate is mediating the transfer of one-carbon moieties (10, 11). In this role, folate is an important factor in DNA synthesis, stability, and integrity, and repair, aberrations of which have been implicated in colorectal carcinogenesis (10, 11). Folate may also modulate DNA methylation (10, 11), which is an important epigenetic determinant in gene expression (an inverse relationship), in the maintenance of DNA integrity and stability, and in the development of mutations (35). A growing body of in vivo and in vitro evidence indicates that folate deficiency is associated with DNA strand breaks, aberrant DNA methylation, impaired DNA repair, and increased mutations and that folate supplementation can correct some of these defects induced by folate deficiency (10, 11). One human study (36) has shown that folinic acid (15 mg/day for 3 months), a reduced and one-carbon-substituted form of folate, significantly reduces colonic epithelial cell proliferation, a purported surrogate end point of carcinogenesis (37), in subjects with chronic UC. Another study (38) has demonstrated that folate supplementation (5 mg/day for 6 months) partially corrects microsatellite instability, the hallmark of DNA mismatch repair defects observed in hereditary and sporadic CRC (39) and UC-associated CRC (40), present in the nonneoplastic colonic mucosa of patients with UC. Lasher et al. (41) have shown that folate supplementation may protect against the development of p53 mutations in subjects with chronic UC.

UC-associated CRC arising in the IL-2null × βmnull mouse is different from the human disease in several aspects. Almost all dysplasia and CRC in this murine model are located in the proximal colon, reminiscent of tumors arising in hereditary nonpolyposis CRC syndrome or microsatellite unstable sporadic CRC. Indeed, our previous molecular analyses have demonstrated that the majority of CRC in the IL-2null × βmnull mice harbor microsatellite instability (23). The frequency and spectrum of Apc mutations are distinctly different from those of human UC-associated and sporadic CRC (23). Nevertheless, the IL-2null × βmnull mouse appears to be an excellent animal model to study chemopreventive effects of dietary factors and potential chemopreventive agents on UC-associated colorectal carcinogenesis for the following reasons: (a) the spontaneous development of colorectal dysplasia and cancer in the setting of chronic mild to moderate or quiescent colitis; (b) clinical and genetic similarities to human UC-CRC; (c) histological similarities to human UC-CRC (i.e., dysplasia to carcinoma sequence); and (d) the accelerated nature of tumorigenesis, which provides an opportunity to determine effects of chemopreventive agents on both initiation and progression of tumorigenesis in a relatively short time. Furthermore, the present study demonstrates that colorectal carcinogenesis in this model can be modulated by a dietary factor.

The observed effects of folate on UC-associated colorectal carcinogenesis in the IL-2null × βmnull murine model need to be confirmed in other animal models of UC-associated CRC (42). Because of the differences between CRC arising in this model and human UC-associated CRC and inherent limitations associated with animal models and observational epidemiological studies, however, the role of folate in UC-associated colorectal carcinogenesis needs to be confirmed in human intervention trials. Given the long time needed for CRC to develop in a surveillance program and the large number of subjects necessary to achieve adequate statistical power and ethical concerns, it would be difficult to conduct randomized, double-blind, placebo-controlled trials in humans. However, with existing standard-of-care colonoscopic surveillance, dysplasia as an end point, and the emergence of several molecular surrogate end point biomarkers, folate chemoprevention trials in individuals with chronic UC at risk of developing CRC are possible and would verify the potential chemopreventive effect of folate observed in animal and observational epidemiological studies.

In summary, our data suggest that dietary folate supplementation at 4 × the basal dietary requirement significantly suppresses UC-associated colorectal carcinogenesis in the IL-2null × βmnull murine model of UC-associated CRC. Our data also suggest that folate deficiency may decrease the incidence of high-grade lesions in chronic UC. However, the high mortality observed in the folate-deficient group precludes a definitive conclusion concerning the effect of folate deficiency on colorectal carcinogenesis in this model. Notwithstanding the limitations associated with this model, data from the present study, in conjunction with epidemiological observations (4–6), suggest that folate chemoprevention of UC-associated CRC in humans merits further consideration. The optimal timing and dose of folate intervention need to be established for safe and effective chemoprevention in humans. Future studies investigating potential mechanisms by which folate supplementation may suppress UC-associated colorectal carcinogenesis in this model are also warranted.

Acknowledgments

We thank Dr. Richard C. Renlund and the veterinary technologists of the Division of Comparative Medicine, University of Toronto for the care of the mice used in this study.

References


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