Folate Deficiency Enhances the Development of Colonic Neoplasia in Dimethylhydrazine-treated Rats

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ABSTRACT

In patients with ulcerative colitis, epidemiological work has suggested an association between low folate status and an increased risk of colonic neoplasia. The aim of the present study was to determine if experimental folate deficiency increases the likelihood of developing neoplasia in rats treated with the carcinogen dimethylhydrazine. Weaning male Sprague-Dawley rats were fed with an amino acid-defined diet containing either 8 or 0 mg/kg folic acid. After 5 weeks of defined diet, weekly s.c. injections of dimethylhydrazine (20 mg/kg) were administered to both groups. Serum, whole blood, liver, and colonic folate concentrations at the time of sacrifice were significantly lower in folate-depleted animals ($P < 0.001$). There were significant differences in the incidence of colonic neoplasia between the two groups after 20 weeks of dimethylhydrazine exposure: folate-deficient rats had a greater incidence of dysplasia (6 of 7 versus 2 of 7 animals; $P < 0.05$) and carcinoma (6 of 7 versus 1 of 7 animals; $P < 0.01$). Furthermore, a significantly greater proportion of folate-replete rats than folate-deficient rats were free of neoplastic lesions (5 of 7 versus 0 of 7 animals; $P < 0.05$).

These results suggest that, in this animal model, folate deficiency increases the risk of malignancy when there is an underlying predisposition to colorectal cancer.

INTRODUCTION

Although a genetic susceptibility to colorectal cancer is well established (1), environmental factors, especially nutrition, are considered to play an important role in most instances (2, 3). A deficiency of the water-soluble vitamin folate is among those nutritional factors which have been considered. The evidence which indicates that folate plays a role in colonic carcinogenesis is indirect at this point: some epidemiological studies suggest an association between folate deficiency and premalignant changes in several epithelial tissues, including the colon (4–7). Lashner et al. (7) have observed that among individuals with ulcerative colitis, an illness which carries with it a substantial risk of folate deficiency, folate supplementation is associated with a 62% reduction in the risk of developing colonic dysplasia or cancer.

The question of whether folate deficiency plays a role in colonic carcinogenesis is an important one because folate deficiency is thought to be the most common vitamin deficiency in the United States as well as the rest of the world (8). Furthermore, folate deficiency is known to complicate several gastrointestinal illnesses in which the risk of developing intestinal malignancies is increased (9–11).

The objective of the present study was to determine whether, and to what extent, folate deficiency enhances the likelihood of developing colonic neoplasia in rats exposed to a well-known colon carcinogen.

MATERIALS AND METHODS

This study was reviewed and approved by the New England Medical Center Animal Research Committee. Ninety-four weanling male Sprague-Dawley rats (60 g, Charles River Co., Wilmington, MA) were randomly divided into four groups. Groups 1 (n = 16) and 3 (n = 31) received a high glutamate amino acid defined diet (12) containing 8 mg/kg of folic acid, whereas groups 2 (n = 16) and 4 (n = 31) received an identical diet except that folate was omitted (Dyets, Inc., Bethlehem, PA). Both diets contained 50 g of cellulose/kg as well as 60% of calories as carbohydrate, 23% as fat, and 17% as $\gamma$-amino acids. No sulfonamide antibiotics were used because the marked folate deficiency associated with sulfonamide use is not consistent with the moderate deficiency state we wished to examine. Furthermore, the use of antibiotics would have interfered with DMH$^4$ activation by bacterial flora (13). Rats were housed individually in wire-bottomed stainless steel cages, diets and water were given ad libitum, and body weights were recorded weekly. After 5 weeks of defined diet groups 3 and 4 received weekly s.c. injections of 20 mg/kg dimethylhydrazine (Sigma, St. Louis, MO) for 20 weeks, while groups 1 and 2 received injections of placebo carrier. DMH was freshly dissolved in 0.05% EDTA and adjusted to pH 6.5 with NaHCO$_3$ as described (14). Three or four animals in groups 1 and 2 and 5–7 animals in groups 3 and 4 were sacrificed at 5, 10, 15, 20, and 22 weeks after the first injection of DMH. One rat in group 4 died at week 5 and was not examined.

The rats were killed by cardiac puncture under methoxyflurane anesthesia. Blood was collected, stored at $-20^\circ$C in 0.5% ascorbic acid, and later assayed for serum and whole blood folate by microbiological assay using Lactobacillus casei (15). One g of liver tissue was rapidly weighed, minced, and added to 10 volumes of 100°C folate extraction buffer (15). After boiling for 10 min, the suspension was cooled to O°C, homogenized, and centrifuged. The clear supernatants were stored at $-70^\circ$C for subsequent assay of total folates. Microbiological assay was carried out using cryoprotected cultures of L. casei after treatment with chicken pancreas conjugate to convert all the polyglutamates to their corresponding monoglutamate derivatives (15). The same procedure was used to measure folate concentration in colonic mucosal scrapings. The values obtained are expressed per gram of tissue.

The entire gastrointestinal tract was examined for the presence of macroscopic tumors. The colon (except for the cecum, which was used for mucosal scrapings) was opened longitudinally, rinsed in saline, and carefully examined for any macroscopic lesions. All such lesions were measured, mapped, and recorded. The colon was then rolled up into a “Swiss roll” (resembling a jelly roll configuration; see Ref. 16), fixed in 10% formalin, and processed for light microscopy. A single longitudinal section (5 $\mu$m) was cut from the midline of each Swiss roll, without regard to the presence of macroscopic lesions, and stained with hematoxylin/eosin. The Swiss roll is a means of providing a pathologist...
with an intact longitudinal section of the entire length of the colon on a single microscope slide, and it has previously been used to quantitatively and qualitatively describe DMH-induced lesions in the colon (16). Since midline sections were cut without regard to the presence of macro- or microscopic lesions, each 5-μm section in this study constituted a random and representative longitudinal section from the entire length of the colon of each rat, excluding the cecum. Assessment of the microscopic lesions was thereby performed in a manner that was independent of the assessment of macroscopic lesions.

Two experienced pathologists read the slides independently and in a blinded fashion. One of the two was designated as the chief pathologist; in those few instances (n = 3) where there was disagreement about the histological interpretation, the opinion of the chief pathologist prevailed. The entire length of each colon, excluding the cecum, was carefully examined by this technique, and each focus of neoplasia was recorded with respect to its location (proximal versus distal) and the severity of dysplasia (vide infra). Histological lesions in colonic mucosa were classified into three categories according to previously described criteria (17, 18): (a) low-grade dysplasia; (b) high-grade dysplasia; and (c) carcinoma. The epithelium was also examined for the presence of megalocytosis and/or megaloblastosis (the former refers to differentiated epithelial cells possessing cytologic characteristics of folate deficiency; the latter refers to undifferentiated stem cells deep within the crypt possessing those characteristics; the remainder of this paper uses the more commonly used term, megaloblastosis, to encompass both cell types). The presence of megaloblastosis was determined qualitatively as previously described (19, 20): epithelial cells possessing nuclei which were larger and more hyperchromatic than normal and whose chromatin pattern was more "open" than normal but whose other cytologic characteristics, such as a columnar shape and basally located nucleus, were retained were considered to be megaloblasic. If such changes were observed anywhere in a Swiss roll, that colon was considered to possess evidence of megaloblastosis.

Statistical significance of the mean values was determined using the two-tailed Student's t test. Nonlinear regression analyses were used to evaluate the association between two variables (MYSTAT; Systat, Inc., Evanston, IL). Fisher's exact test was used to determine the significance of differences in the proportion of neoplastic lesions observed in groups 3 and 4.

RESULTS

Body Weight. There were no significant differences in the growth curves of animals fed a folate-deficient or folate-replete diet. However, DMH-injected animals (groups 3 and 4) were significantly lighter when compared to non-DMH-injected animals (groups 1 and 2) from the 13th week on (P < 0.05) (Fig. 1).

Folate Status. Table 1 shows that systemic folate deficiency was clearly present in groups 2 and 4 as demonstrated by serum, whole blood, and liver folate concentrations. The table also demonstrates that folate depletion was present in the colonic mucosa itself. Progression of folate deficiency was not observed after the fifth week of DMH or placebo injections, which is the first time point at which rats were sacrificed. For example, serum folate concentration, which is considered to be an accurate indicator of systemic folate status in rats by Clifford et al. (21), did not change significantly between the first time point (5 weeks) and the last time point (22 weeks) in any of the 4 groups of animals. Accordingly, the average values displayed in Table 1 represent the pooled values from all of the rats in each of the four groups.

Macroscopic Data. No neoplastic lesions were observed in non-DMH-injected animals (groups 1 and 2). Also, there were no small intestinal or extraintestinal malignancies observed in DMH-injected animals. No metastases were observed in any of the groups. The cecum was everted and carefully examined in each rat before it was processed for biochemical analysis; no macroscopic lesions were observed.

Macroscopically evident DMH-associated lesions appeared as either pedunculated or sessile polyps, as previously described (22, 23). In both groups the size of the lesions ranged from 2 to 20 mm. At 20 weeks, a greater proportion of group 4 had polyps than group 3, although the difference was not statistically significant (6 of 7 or 86% versus 3 of 7 or 43%; P = 0.09). The manner in which the colons were processed in this study precluded histological analysis of macroscopic lesions. Nevertheless, previous investigators who have used this animal model have observed that macroscopic lesions invariably contain neoplastic foci (16, 24-28), establishing the assessment of macroscopic lesions as a reflection of neoplastic transformation.

Microscopic Data. Megaloblastosis was observed in 15% (2 of 16) of the animals in group 2, in 40% (12 of 30) of the animals in group 4, and in none of the animals in groups 1 and 3. The cancers were well-differentiated adenocarcinomas in all instances except in one case in group 4, where a signet ring-type carcinoma was observed. Most of the highly neoplastic lesions (carcinoma and high-grade dysplasia) were located adjacent to hypertrophic lymphoid follicles.
Fig. 2 presents the percentage of DMH-injected rats that had either dysplasia and/or carcinoma and gives an indication of the time course of developing neoplastic lesions in this study. As is indicated in the figure, the earliest neoplastic lesions in DMH-injected rats were observed at 15 weeks. Although the folate-deficient animals had a higher prevalence of neoplasia at this time point, the differences between the two groups were not significant. However, highly significant differences in the prevalence of neoplasia were observed by 20 weeks ($P < 0.01$). Table 2 summarizes the histological findings in DMH-injected rats at 20 weeks; by this time point a significantly greater proportion of the folate-deficient animals were observed to have dysplasia ($P < 0.05$) and carcinoma ($P < 0.01$). Furthermore, the proportion of folate-replete animals that were free of any neoplastic lesions at 20 weeks was significantly greater than the proportion of neoplasia-free animals in the folate-deficient group (5 of 7 versus 0 of 7, respectively; $P < 0.05$). Table 3 lists the individual neoplastic lesions observed in each of the DMH-injected rats at 20 weeks; the table also specifies whether each lesion was located in the proximal or distal half of the colon and whether each dysplastic lesion was high grade or low grade. There were no statistically significant differences between groups 3 and 4 with respect to the preponderance of proximal versus distal lesions or with respect to the proportion of dysplastic foci which were high-grade lesions.

At the doses of DMH used in this study all animals would be expected to eventually develop colon cancer. This was found to be true; by 22 weeks all of animals in groups 3 and 4 had developed dysplasia and/or carcinoma. At this time point, only 5 rats in group 3 and 4 rats in group 4 were sacrificed. There was a trend toward a greater number of neoplastic lesions per rat in the folate-deficient group (4.8 versus 2.5 lesions/rat, group 4 versus group 3, respectively) but the relatively small number of rats at this time point precluded any meaningful statistical analysis.

**DISCUSSION**

This study demonstrates that moderate folate deficiency enhances the development of DMH-related neoplasia in the rat colon. Several clinical studies have suggested an association between folate deficiency and dysplasia in the cervical (4), bronchial (5, 6), and colonic (7) epithelium. In addition, some of these studies have demonstrated an apparent reversal of the pathologic lesions with supplemental folate (4, 6). However, no clinical study to date has been able to establish a clear cause-and-effect relationship between folate deficiency and dysplasia. The present study provides strong evidence for such a relationship: the experimental animals, which clearly had both systemic and colonic folate deficiencies, showed an enhanced susceptibility to the effects of a colon carcinogen compared to a control group consisting of folate-replete animals. It is worth emphasizing that the procarcinogenic effect of folate deficiency that we observed was achieved with a diet that produces a modest degree of deficiency that is not severe enough to cause growth retardation or anemia (12). This contributes to the potential clinical significance of these observations, since mild folate deficiency in humans is much more common than a severely deficient state.

It is also important to point out that group 2 animals did not develop any neoplastic changes; this confirms the concept that folate deficiency is not carcinogenic by itself (29). However, when superimposed on an underlying predisposition to cancer, folate deficiency seems to increase the susceptibility to neoplastic transformation. Such a relationship is underscored by gastrointestinal illnesses, such as ulcerative colitis and celiac sprue, which are associated with both an increased risk of folate deficiency and an increased susceptibility to cancer (9-11, 30, 31).

A significant problem with some of the above-mentioned clinical studies exists because of a confusing similarity between the cytological characteristics of dysplastic and megaloblastic cells. In the trials involving folate supplementation (4, 6), the
similarity between the two cell types confounds the interpretation of whether dysplasia or megaloblastosis regressed with therapy (6). In the present study, animals in groups 2 and 3 enabled us to observe cases which were either solely megaloblastic or solely dysplastic. The fact that no megaloblastosis was observed in group 3 and no dysplasia in group 2 suggests that our blinded pathologists were capable of objectively distinguishing between these two cell types. Nevertheless, the prevalence of observed megaloblastosis in group 4 was greater than in group 2 (40% versus 15%). Since the degree of folate deficiency was similar in the two groups, this observation raises the question of whether some of the lesions observed in group 4 animals which were classified as megaloblastosis were in fact dysplasia or whether there is a more complicated functional relationship between these two cell types which is not yet fully understood.

Determining whether folate deficiency was present in the colonic mucosa was an important feature of this study, since some tissues appear to be more resistant to depletion than others (21). The cecum was chosen as a representative portion of the colon for folate analysis. Given the substantial degree of folate deficiency observed in the cecum and in the other tissues which were examined in the folate-deficient rats, it is unlikely that the remainder of the colon would have been folate replete in these animals. The fact that the cecum was used to obtain mucosal tissue for folate analysis precluded histological analysis of this portion of the colon. Therefore, by necessity, our conclusions regarding the procarcinogenic effect of folate deficiency are limited to that portion of the colon distal to the cecum. It is unlikely that excluding the cecum from the histological analysis substantially skewed our observations, particularly since we observed that the geographic distributions of neoplasia in groups 3 and 4 were not significantly different.

The mechanism by which local folate deficiency might contribute to the risk of malignant changes remains unclear. However, it is worth noting that folate is a critical element for thymidylic acid synthesis through thymidylate synthetase (29, 32). Thymidine deprivation has been shown to result in profound alterations in nucleotide pools (33), which will result in a reduction in the rate of DNA replication (34). In addition, reduction of the deoxythymidylate pool leads to expansion of the deoxyuridylate pool and abnormally high incorporation of uracil into DNA. The repair of misincorporated uracil under conditions of folate deficiency results in the so-called catastrophic repair in which further incorporation of uracil may take place (35). It has been postulated that this interference with the biosynthesis of deoxynucleotides is responsible for the procarcinogenic effect of folate deficiency (29). In the present study nucleotide pools were not measured. However, prior studies by us indicate that folate deficiency in rats on a diet identical to that used in this study leads to an impairment of thymidylate-DNA synthesis in the colonic epithelium (36). Another possible explanation why folate-deficient cells apparently have a greater susceptibility to undergo neoplastic transformation is related to DNA methylation. Rogers and Newberne (37) demonstrated that diets deficient in methionine, choline, folate, and vitamin B12 (lipotropes) promote chemical carcinogenesis in rats. This carcinogenic effect seems to be related to a decrease in the concentration of S-adenosylmethionine that occurs as a consequence of dietary methyl deprivation (38). S-adenosylmethionine serves as a methyl donor for many biochemical reactions, including DNA methylation (29, 39). Although the role of DNA methylation in gene regulation is not fully understood, many studies have shown that a decrease in methylation is associated with gene activation (40, 41). Previous studies have suggested that generalized DNA hypomethylation as well as specific protooncogene hypomethylation precedes the development of malignancy in both experimental and human colonic tumors (42-44). Since folate is a necessary cofactor for the regeneration of S-adenosylmethionine, it is possible that folate deficiency results in hypomethylation of DNA and activation of specific protooncogenes.

Given the high incidence of colorectal cancer in the general population and the substantial prevalence of folate deficiency in certain segments of our population (8), these observations raise the question of whether maintenance of adequate folate status in patients at high risk for colorectal cancer may be an important factor in minimizing the risk of developing colonic neoplasia. Further studies will be necessary to confirm our observations in experimental carcinogenesis and in order to establish whether, and under what conditions, folate deficiency might enhance colon carcinogenesis in humans.

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