Dietary folate protects against the development of macroscopic colonic neoplasia in a dose responsive manner in rats

Young-In Kim, R N Salomon, F Graeme-Cook, Sang-Woon Choi, D E Smith, G E Dallal, J B Mason

Abstract

Background and Aims—Diminished folate status is associated with enhanced colorectal carcinogenesis. This study investigated the potential chemopreventive role of dietary folate in the dimethylhydrazine colorectal cancer model.

Methods—Sprague-Dawley rats were fed diets containing either 0, 2 (daily dietary requirement), 8 or 40 mg folate/kg diet for 20 weeks. After five weeks of diet, rats were injected with dimethylhydrazine (44 mg/kg) weekly for 15 weeks. Fifteen weeks after the first injection of dimethylhydrazine, all rats were killed. Folate status was determined, and the entire colorectum from each rat was analysed for macroscopic and microscopic neoplasms.

Results—Plasma and colonic folate concentrations correlated directly with dietary folate levels (p<0.005). The incidence of microscopic neoplasms was similar among the four groups. However, the incidence and the average number of macroscopic tumours per rat decreased progressively with increasing dietary folate levels up to 8 mg/kg diet (p<0.05). In the strongly procarcinogenic milieu used in this study, folate supplementation at 20 times the basal requirement was associated with rates of macroscopic tumour development that were intermediate, and not statistically distinct, from rates observed at either 0 or 8 mg/kg diet.

Conclusions—These data indicate that in this rat model, (a) increasing dietary folate up to four times the basal requirement leads to a progressive reduction in the evolution of macroscopic neoplasms from microscopic foci; and (b) folate supplementation beyond four times the requirement does not convey further benefit.

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Keywords: colorectal cancer, chemoprevention, folate, dimethylhydrazine, DNA methylation.

Although genetic alterations have been shown to play an important part in colorectal carcinogenesis, it has been estimated that up to 90% of colorectal cancer in the United States can be attributed to dietary factors. Much effort, therefore, has been directed towards defining the relation between nutritional factors and the development of colorectal cancer, and towards the prevention of colorectal cancer through dietary modifications.

Recently, considerable interest has been focused on folate because diminished status of the vitamin has been shown in epidemiological and animal studies to be associated with an increased risk of neoplastic transformation. Epidemiological studies conducted in people with ulcerative colitis, as well as in the general population, indicate a 30–50% reduction in colorectal neoplasia among those with higher, rather than lower, folate status. The most convincing evidence that diminished dietary folate intake is associated with an increased risk of colorectal neoplasia has been recently published by Giovannucci et al. This prospective study included over 25,000 cohorts and correct for the most of the well known dietary confounders associated with colorectal cancer. The study observed a 40% reduction in the risk of developing colorectal adenomas in subjects with the highest amount of dietary folate intake compared with those with the lowest intake. More recently, prospectively conducted clinical studies have shown that red blood cell or colonic mucosal folate concentrations are significantly lower in those harbouring adenomas compared with control subjects, thereby supporting epidemiological findings.

In support of the causal inferences suggested by epidemiological studies, previous work in the dimethylhydrazine (DMH) rodent model of colorectal cancer has shown that moderately folate deplete rats have a significantly greater incidence of microscopic dysplasia and invasive cancer than folate replete controls. The DMH rodent model of colorectal carcinogenesis mimics the histopathology and molecular biology of the human disease in many respects, and it has been extensively used in studies that have examined the modulatory effect of nutritional factors on the development of colorectal cancer.

To date, the exact mechanisms by which diminished folate status increases colorectal carcinogenesis have not been clearly elucidated, although several candidates have been proposed. Folate is an essential factor for a number of critical metabolic pathways in the cell that involve the transfer of one carbon groups. Among such pathways are the synthesis of S-adenosylmethionine (SAM), the methyl group donor for most biological transmethylation reactions, including that of...
Dietary folate protects against the development of colonic neoplasia

Dietary folate has been shown to have an effect on DNA methylation at cytosine-guanine dinucleotides (CpG), an epigenetic modification of DNA that is observed in several human and experimental cancers. Similar effects are observed in certain protooncogene specific DNA hypomethylation has been observed in early stages of colorectal carcinogenesis. Although studies examining the effect of folate deficiency on genomic DNA methylation remains an unsettled issue, folate supplementation at 50 times the daily requirement for humans has recently been shown to be capable of increasing the extent of genomic DNA methylation of the colonic mucosa in subjects with colorectal adenocarcinoma or adenoma.

Previous work in this animal model has merely established that rats moderately deficient in folate develop microscopic neoplasms at a greater rate than rats receiving modest supplementation with the vitamin. The objectives of this study were to find out if the process by which microscopic neoplasms evolve from microscopic foci is also suppressed by folate supplementation and to define the dose response nature of this relation. In addition, we investigated the effect of folate deficiency and supplementation, in conjunction with DMH administration, on colonic mucosal SAM concentrations and genomic DNA methylation because such changes may play a mechanistic part in mediating the enhancement of colorectal carcinogenesis by folate insufficiency.

Methods
This study was approved by the institutional Animal Care and Use Committee of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University. Forty weaning male Sprague-Dawley rats (60–90 g, Charles River Co, Wilmington, MA) were randomly assigned to receive an amino acid defined diet (Dyets, Bethlehem, PA) containing either 0, 2, 8 or 40 mg folate/kg diet (n=10, each group). Succinylsulphathiazole, which is conventionally used to create a severe folate deficiency, was not incorporated into the diets in this study for several reasons. Firstly, we wish to avoid severe folate deficiency, which predictably causes severe growth retardation and premature death; secondly, the consensus of epidemiological and clinical studies and our previous animal study indicate that mild to moderate depletion of folate is sufficient to increase colorectal carcinogenesis; and thirdly, the use of antibiotics interferes with DMH activation. The amino acid defined diets in this study were identical to those used in a previous study in which rats receiving 0 mg folate/kg diet demonstrated a greater incidence of microscopic foci of colonic neoplasia compared with those receiving 8 mg folate/kg diet. Two mg folate/kg diet is generally accepted as the basal dietary requirement for the rat. Forty mg folate/kg diet (that is, 20 times the basal requirement) was chosen in this study because a recent clinical trial observed that folate supplementation in the amount of 50 times the daily requirement for humans was able to modulate a biomarker of colon cancer in humans. These diets contained 50 g of cellulose/kg as well as 60% of calories as carbohydrate, 23% as fat and 17% as L-amino acids. Rats were housed individually in wire bottomed stainless steel cages to minimise coprophagy. Diets and water were supplied ad libitum. Body weights were recorded weekly. The average daily food consumption of each group was determined on a pre-determined day of each week. Five weeks after diet initiation, rats were injected subcutaneously with 44 mg DMH.2HCl (Sigma, St Louis, MO) per kg body weight weekly for 15 weeks. This dose of DMH is higher than the conventional dose used in induction of colorectal neoplasia in this species of rat and was deliberately chosen to create a potent procarcinogenic milieu to examine the effect of folate supplementation on the progression of microscopic neoplastic foci to macroscopic tumours. DMH was freshly dissolved in 0-05% EDTA and adjusted to pH 6-5 with NaHCO3, as described. All rats were killed 15 weeks after the first injection of DMH (that is, after 20 weeks of the defined diets) by exsanguination under carbon dioxide anaesthesia.

At the time of death, blood was withdrawn from the inferior vena cava using a pre-heparinised 18 gauge needle into vacutainer tubes containing EDTA and centrifuged at 800×g for 10 minutes at 4°C. Plasma was stored at −70°C in 0-5% ascorbic acid for the plasma folate assay. One hundred microlitre aliquots of plasma were stored without ascorbate for homocysteine assays. Blood samples for complete blood counts were collected into tubes containing sodium EDTA and analysed immediately (System 9000; Serono Baker Diagnostic, Allentown, PA).

The entire colorectum from the caecum to the anus was excised and put on a glass plate suspended on crushed ice. It was then opened longitudinally, rinsed in 0-9% NaCl, and examined in a blinded fashion for any macroscopic lesions, as defined as any masses or nodules with vascular abnormalities, under a dissecting microscope. All such lesions were mapped and their largest diameter was measured and recorded. Any submucosal thickening without vascular abnormalities (lymphoid follicles) were also measured and mapped. The colorectum was then sectioned longitudinally into two halves of equal width. The mucosal layer from one section was carefully removed by scraping with glass slides. One half of the resulting mucosal scrapings was rapidly weighed, frozen in liquid nitrogen, and stored at −70°C for subsequent analysis of colorectal mucosal folate concentrations and DNA extraction. The other half of the mucosal scrapings was immediately homogenised in two volumes of 0·4 M perchloric acid and centrifuged at 1500×g for 10 minutes, and then the resulting supernatant was frozen at −70°C for subsequent analysis of colonic SAM and S-adenosylhomocysteine (SAH).

The other section was rolled up into a ‘Swiss roll’, a means by which an intact longitudinal
section of the entire length of the colorectum can be presented on a single microscope slide, and fixed in 10% formalin, and processed for light microscopy. A single longitudinal section (5 μm) was cut from the midline of each Swiss roll, without regard to the presence of macroscopic lesions, and stained with haematoxylin and eosin. As midline sections were cut without regard to the presence of macroscopic 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. This method has previously been used to quantitatively and qualitatively describe DMH-induced lesions in the colorectum. Assessment of the microscopic lesions were 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. Two separate readings were obtained to assess concordance, thereby establishing a measure of objectivity in the histopathological analysis. The entire length of each colon was carefully examined for megaloblastic changes and neoplastic foci. Each focus of neoplasia was recorded with respect to its location (proximal vs. distal) and was classified into low or high grade dysplasia (LGD and HGD, respectively) or invasive adenocarcinoma according to previously described criteria. The presence of megaloblastosis was determined qualitatively as previously described: epithelial cells possessing nuclei that were larger and more hyperchromatic than normal and whose chromatin pattern was more ‘open’ than normal but whose other cytological characteristics, such as columnar shape and basally located nucleus, were retained were considered to be megaloblastic.

Plasma folate concentrations were measured by a microtitre plate assay using *Lactobacillus casei*. Colonic mucosal folate concentrations were measured by the same microbiological assay after extracting in four volumes of fresh folate extraction buffer (5 mM β-mercaptoethanol and 0.1 M sodium ascorbate in 0.1 M (bis 2-hydroxyethyl) imino TRIS [hydroxymethyl]-methane, pH 7.8) followed by treatment with chicken pancreas conjugase to convert all the polyglutamates to their corresponding monoglutamate derivatives. Total plasma homocysteine was measured by high performance liquid chromatography (HPLC) according to the fluorometric method of Vester and Rasmussen. This method incorporates a treatment of the samples with a reducing agent (mercaptoethanol) before analysis and, therefore measures the sum of free, protein bound and disulphide forms of homocysteine. Concentrations of colonic mucosal SAM and SAH were determined by HPLC with ultra violet detection.

The DNA from colonic mucosal scrapings were extracted by a standard technique using a lysis buffer containing proteinase K followed by phenol, chloroform, and isoamyl alcohol organic extraction. The resulting DNA was precipitated with 1 M NaCl and 100% ethanol and was treated with RNase. The purified DNA was further dialysed against 10 mM TRIS-HCl, 1 mM EDTA, pH 8.0. The size of DNA estimated by agarose gel electrophoresis was >20 kb in all instances. No RNA contamination on agarose gel electrophoresis. The final preparations had an A260/280 ratio >1.8. The purified DNA was stored at −70°C until the DNA methylation assay.

Genome wide methylation status at CpG from colonic mucosal DNA was determined by the in vitro methyl acceptance capacity of DNA using [3H-methyl] SAM as a methyl donor and a prokaryotic CpG DNA methyltransferase as previously described and validated. The manner in which this assay is performed produces a reciprocal relation between the endogenous DNA methylation status and the exogenous 3H-methyl incorporation. Briefly, two μg of DNA were incubated in 5 μCi of [3H-methyl] SAM (New England Nuclear Boston, MA; 3–10 Ci/mmol), four units of Sss1 methylase (New England Biolabs, Beverly, MA), one volume Sss1 buffer (50 mM NaCl, 10 mM TRIS-HCl, 10 mM EDTA, 1 mM dithiothreitol, pH 8.0) in a total volume of 50 μl methylation mixture for three hours at 37°C. Sss1 was denatured by heating at 65°C for 20 minutes. The incubation mixtures were applied onto discs of Whatman DE-81 ion exchange filters (Fisher Scientific Springfield, NJ) using vacuum filtration apparatus, and the discs were then washed with 5% NaH2PO4, for 45 minutes. The discs were then dried at 95°C for 30 minutes and the resulting radioactivity of the DNA retained in the discs was measured by scintillation counting using a non-aqueous scintillation fluor. The amount of radiolabel bound to a filter from an incubation mixture lacking DNA was used as a background and was subtracted from the values obtained with mixtures containing DNA. The background value was always <1% of the uptake observed with DNA samples. All analyses were done in duplicate.

For the statistical analyses, the entire population of rats (n=10) from each group were analysed. However, for other biochemical and molecular analyses, adequate amounts of plasma and colonic tissue were not available from one rat from the folate deplete group and hence this rat was excluded from these analyses.

For continuous response variables, differences among dietary groups were assessed by using single factor analysis of variance. When the overall F ratio achieved statistical significance, orthogonal polynomial contrasts, after a square root transformation of dietary folate, and Fisher’s least significant difference were used to better understand the nature of the between group differences. For discrete response variables, differences among groups were assessed by using a Kruskal-Wallis non-parametric analysis of variance; the Jonkheere-Terpstra test was used to test for trend in such instances. For categorical response variables, differences among groups were assessed by Fisher’s exact test and Wilcoxon’s rank sum test was used to test for trend. Continuous
response variables were analysed by using SYSTAT, version 5.03 (SPSS Inc, Chicago, IL). Discrete and categorical variables were analysed by using StatXact for SYSTAT, version 1.01 (Cytel Software Corp, Cambridge, MA). All statistical tests were two sided and were considered statistically significant if the observed significance level (p value) was less than 0.05. Results are expressed as mean (SEM).

Results

Body weight and average daily food consumption

Growth curves were similar in the four groups of rats; at no time point did the mean weights differ significantly among these groups. No premature death occurred. This finding indicates that the folate deficiency in the rats that were fed 0 mg folate/kg diet was not severe, otherwise growth retardation or premature death would have occurred.\(^\text{29}\) The average daily food consumptions, which were determined on a pre-assigned day of each week were also similar among the four groups (data not shown).

Folate and SAM status

As Figure 1 shows, the mean plasma and colonic mucosal folate concentrations were different among the four groups (p<0.001, ANOVA) and correlated directly with the levels of dietary folate (p<0.001 and p=0.003, respectively). However, colonic folate concentrations reached a plateau beyond four times the basal requirement of folate. This finding is probably related to the fact that folate accumulation in tissues is limited by the level of folypolyglutamate synthetase activity in the setting of substrate excess\(^\text{41-43}\) and is consistent with previous studies in animals and in cultured cells.\(^\text{41-43}\) A highly significant correlation between plasma and colonic mucosal folate concentrations was observed (r=0.54, p=0.001).

Plasma concentrations of homocysteine, which are known to rise in the setting of folate deficiency,\(^\text{44}\) were increased by 70% in the folate depleted rats compared with the mean value of the three groups receiving dietary folate (7.26 (0.70) nmol/ml vs 4.36 (0.32), p=0.003). The mean plasma concentrations among the three groups receiving dietary folate were similar (4.44 (0.63) nmol/ml, 4.30 (0.59), and 4.34 (0.47), respectively). This is consistent with previous studies that have suggested that folate supplementation at four times the dietary requirement does not lower plasma homocysteine concentrations beyond the level achieved by dietary folate at the daily requirement.\(^\text{39}\) Individual plasma homocysteine values were inversely correlated with the levels of dietary folate (r=-0.27, p=0.04), plasma folate concentrations (r=-0.39, p=0.013), and colonic mucosal folate concentrations (r=-0.36, p=0.025).

Haemoglobin, packed cell value, and mean corpuscular volume were not significantly different among the four groups (data not shown).

Colonic mucosal concentrations of SAM as well as SAH, the second of which accumulates as a result of folate deficiency and which indicates a decreased ability to remethylate homocysteine,\(^\text{23}\) were not significantly different among the four groups (data not shown). Furthermore, the ratio of SAM to SAH, considered by some as a better indicator of methylating capacity,\(^\text{45}\) was similar among the four groups (data not shown).

Microscopic lesion analysis

The two independent analyses of microscopic lesions by two pathologists were highly correlated: LGD, r=0.3, p=0.01; HGD, r=0.6, p<0.001; and invasive cancer, r=0.9, p<0.001. Furthermore, all analyses pertaining to microscopic data had identical outcomes regardless of which histopathological data set were used (data not shown). Having thereby demonstrated a high degree of concordance, the readings from one of the pathologists were used and reported for all subsequent analyses reported in this paper. Methylalbogenic changes in the colonic epithelium were observed in 80%, 60%, 70%, and 40% of the rats receiving 0, 2, 8, and 40 mg folate/kg diet, respectively. This is consistent with earlier reports that DMH, without the superimposition of folate deficiency, may produce changes simulating methylalbogenic.\(^\text{20}\) The cancers were adenocarcinomas in all instances.

The proportion of rats with LGD, HGD, invasive cancer or any combination thereof was similar among the four groups (Table). Similarly, the proportion of rats with multiple microscopic lesions, in various combinations,
Percentage of rats with microscopic neoplastic foci in four study groups that received four different levels of dietary folate

<table>
<thead>
<tr>
<th>Amount of dietary folate (mg/kg diet)</th>
<th>0 (n=10)</th>
<th>2 (n=10)</th>
<th>8 (n=10)</th>
<th>40 (n=10)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low grade dysplasia (LGD)</td>
<td>80</td>
<td>90</td>
<td>80</td>
<td>70</td>
<td>0.74</td>
</tr>
<tr>
<td>High grade dysplasia (HGD)</td>
<td>10</td>
<td>20</td>
<td>50</td>
<td>30</td>
<td>0.22</td>
</tr>
<tr>
<td>Invasive cancer (CA)</td>
<td>20</td>
<td>30</td>
<td>10</td>
<td>20</td>
<td>0.74</td>
</tr>
<tr>
<td>LGD or HGD</td>
<td>80</td>
<td>90</td>
<td>80</td>
<td>70</td>
<td>0.74</td>
</tr>
<tr>
<td>LGD or HGD or CA</td>
<td>90</td>
<td>90</td>
<td>80</td>
<td>70</td>
<td>0.59</td>
</tr>
</tbody>
</table>

was not statistically different among the four groups (data not shown). A similar analysis, which examined the average number of neoplastic foci per animal, also showed no significant differences in the burden of microscopic foci in the four groups (data not shown). No significant correlation was observed between plasma or colonic folate concentrations and the presence or the number of microscopic neoplasms. Colonic SAM concentrations and SAM/SAH ratios were not significantly correlated with the presence or the number of microscopic lesions.

**Macroscopic tumour analysis**

Most of the macroscopic tumours (90%) were located in the distal colon. Non-specific submucosal fold thickening without vascular abnormalities were present in a similar proportion of rats in each group and most of these (91%) were located in the proximal colon. Two rats had hepatic, and peritoneal and omental metastatic lesions, respectively: both were in the group receiving 0 mg folate/kg diet.

As Figure 2 shows, the proportion of rats harbouring macroscopic tumours decreased progressively with increasing levels of dietary folate up to 8 mg folate/kg diet (p=0.029, Fisher’s exact test; p=0.011, Wilcoxon test for trend). With dietary folate supplementation at 20 times the daily requirement, there was no further decrease in the proportion of rats with macroscopic tumours compared with the group receiving 8 mg folate/kg diet (p=0.2, Fisher’s exact test). In fact, a non-significant trend towards a larger proportion of rats with macroscopic neoplasms was evident in the 40 mg/kg group compared with the 8 mg/kg group.

Similarly, Figure 3 shows that the average number of macroscopic tumours per rat decreased progressively with increasing levels of dietary folate up to 8 mg folate/kg diet (p=0.036, Kruskal-Wallis; p=0.016 Jonkheere Terpstra test for trend) and that a non-significant trend towards greater numbers of macroscopic tumours per rat was evident in the group receiving 40 mg folate/kg diet compared with those receiving 8 mg folate/kg diet.

The size of the macroscopic tumours ranged from 2.0 mm to 6.7 mm. The average size of the macroscopic tumours were not significantly different among the four groups, although a trend similar to the incidence and the average number of macroscopic tumours per rat was observed (3.9 (0.7) mm, 3.5 (0.1), 2.0, and 2.9 (0.4) in the rats receiving 0, 2, 8 and 40 mg folate/kg diet, respectively).

**Genome wide colonic DNA methylation status**

The extent of genome wide colonic DNA methylation was not statistically different among the four groups (274 806 (34 601), 248 142 (29 954), 235 804 (26 074), and 247 438 (24 239), respectively). Furthermore, the degree

![Figure 2: Proportion of rats with macroscopic tumours in the four groups of rats fed different levels of dietary folate (n=10 in each group). The proportion of rats with macroscopic tumours decreased progressively with increasing levels of dietary folate up to 8 mg folate/kg diet (p=0.029, Fisher’s exact test; p=0.011, Wilcoxon test for trend). Folate ‘hypersupplementation’ exceeding four times the basal requirement did not convey further inhibition of tumorigenesis.](http://gut.bmj.com/)

![Figure 3: Average number of macroscopic tumours per rat in the four groups of rats receiving different levels of dietary folate (n=10 in each group). The average number of macroscopic tumours per rat decreased progressively with increasing levels of folate up to 8 mg folate/kg diet (p=0.036, Kruskal-Wallis, p=0.016, Jonkheere-Terpstra test for trend). Folate ‘hypersupplementation’ exceeding four times the basal requirement did not convey further inhibition of tumorigenesis.](http://gut.bmj.com/)
of genomic methylation was not correlated with either plasma and colonic folate concentrations, colonic SAM concentrations or SAM/SAH ratios or plasma homocysteine levels.

Those rats harbouring macroscopic tumours had a significantly higher mean level of [3H] methyl group incorporation into DNA compared with those without (288,480 (25,012) dpm v 224,844 (14,078); p=0.03), indicating a significantly lower extent of genomic DNA methylation in the rats bearing macroscopic tumours.

Discussion

This study shows that folate modulates the process of colorectal carcinogenesis over a wide range of dietary intakes in this rodent model of colon cancer, thereby confirming previously published observations from epidemiological, clinical, and animal studies. In particular, these data indicate that a folate deficient diet is associated with a potentiation of the development of macroscopic neoplasms from microscopic neoplastic foci in a dose responsive manner. One interesting finding from this strongly procarcinogenic model is that levels of dietary folate beyond four times the dietary requirement, leads to a progressive reduction in the evolution of macroscopic neoplasms from microscopic neoplastic foci in a dose responsive manner. One interesting finding from this strongly procarcinogenic model is that levels of dietary folate beyond four times the dietary requirement, leads to a progressive reduction in the evolution of macroscopic neoplasms from microscopic neoplastic foci in a dose responsive manner. However, because the dose of DMH used in this study was considerably higher than the conventional dose used in induction of colorectal neoplasia, the promoting effect of DMH may have overwhelmed the effect of folate and thus, the magnitude of the folate effect could have been underestimated.

In our previous study, 100% of the rats that were fed 0 mg folate/kg diet developed microscopic neoplasia 20 weeks after receiving DMH (20 mg/kg body weight) injections compared with 29% of the rats that were fed 8 mg folate/kg diet (p<0.005). In contrast, the proportion of macroscopic tumours between the folate depleted and control groups was not significantly different (86% v 43%, p=0.09).

The earlier study, therefore, suggested that folate deficiency acts on an early phase of DMH induced colorectal carcinogenesis. In the present study, the dose of DMH (44 mg/kg body weight) was more than twice the dose used in the previous study, and was deliberately chosen to create a very strong procarcinogenic milieu to elucidate more effectively the effect of folate supplementation on the progression of microscopic neoplastic foci to macroscopic tumours. As the induction of colonic neoplasms by DMH is dose dependent, not surprisingly, almost all rats had microscopic neoplastic foci, independent of the levels of dietary folate, at a time point that was five weeks earlier than that of the previous experiment. However, this study clearly demonstrates that folate supplementation renders protection against the evolution of microscopic neoplastic foci to macroscopic tumours in a dose responsive manner. Taken together, these data suggest that folate insufficiency or supplementation has modulatory effects on the development of colorectal neoplasms in rats injected with DMH and that the exact stages of tumorigenesis on which it has the most profound effect seems to be related to the DMH doses used. The schedule used in this study does not permit us to distinguish whether the modulatory effects of dietary folate are exerted on the initiation or promotional stages of carcinogenesis: future studies are warranted to consider this issue.

The degree of folate depletion observed in the rats fed 0 mg folate/kg diet was moderate as evidenced by the plasma and colonic mucosal folate concentrations as well as the absence of growth retardation and premature death. Though the folate depletion was only moderate, there was evidence of biochemically significant folate deficiency, as indicated by the modest rise in plasma homocysteine concentrations compared with those of the folate replete rats. This is consistent with other observations that indicate that this magnitude of folate depletion is known to be associated with functionally significant degree of cellular folate depletion within the colonic mucosa. Therefore, the finding that even a mild to moderate degree of folate depletion can potentiate the action of DMH on the development of macroscopic tumours in rats further substantiates the epidemiological and clinical studies that have suggested that a modest reduction in folate status is all that is necessary for enhancement of colorectal carcinogenesis when other risk factors are present. Moreover, the fact that those rats fed the daily requirement of folate had a higher incidence of macroscopic tumours compared with those supplemented with folate at four times the daily requirement is consistent with observations from some of the epidemiological studies where the risk of developing colorectal cancer or its precursor, adenoma, is decreased in subjects taking higher amounts of dietary folate than the tumours between the folate depleted and control groups were not significantly different (86% v 43%, p=0.09).

Homocysteine is significantly increased in the folate depleted animals compared with the replete groups even though colonic mucosal SAM, SAH, and SAM/SAH ratio are not significantly changed in this group. Interestingly, colonic SAH is not increased in the folate depleted animals even though homocysteine accumulation favours a backwards reaction whereby SAH is created. These observations are consistent with an earlier study where SAM and SAH from the liver and colonic mucosa were compared in rats receiving 0 and 8 mg folate/kg diet. In that study, plasma homocysteine concentrations were threefold higher in the folate depleted rats compared with the folate replete animals, an effect that was present in association with a significant increase in hepatic SAH and significant decrease in hepatic SAM. However, in the earlier study, as is true in this one, no significant changes in colonic SAM and SAH were observed. This is probably related to the fact
that the degree to which one carbon metabolism is affected by folate depletion is highly tissue dependent.\textsuperscript{36} Furthermore, it reflects the fact that homocysteine is distributed in the body in a highly compartmentalised and heterogeneous fashion\textsuperscript{46} and therefore an accumulation of homocysteine in the plasma does not necessarily imply its accumulation in the colonic mucosa.

We observed a highly significant positive correlation between the plasma concentration of folate and the concentration of folate in the colonic mucosa; this is consistent with earlier studies in rats that observed similarly strong correlations between serum folate levels and folate levels in the gastrointestinal tract.\textsuperscript{46} Nevertheless, in a recent study in humans,\textsuperscript{37} no such correlation was observed between either plasma or red blood cell folate concentrations and concentrations in isolated colonocytes. In the instance of this second study, colonocytes were first isolated and allowed to equilibrate for several hours before the determination of folate content. In contrast, our method stabilises the folate content at the time of tissue isolation and is furthermore designed to determine the folate concentration of all the contents of colonic mucosa, including epithelial mucous and cells from the lamina propria, both of which are important constituents of the environment in which colonic epithelial cells exist. Therefore, the apparent discrepancy between our study and the abovementioned human study maybe ascribable to species differences or methodological differences: future studies should help clarify this issue.

The observations in this study indicate that the significant, and progressive, decline in colorectal neoplasia that is observed with increasing levels of dietary folate does not continue at levels of folate that exceed four times the daily requirement. The fact that there is no significant difference in the incidence of macroscopic neoplasia between the 8 and 40 mg/kg groups (see Figs 2 and 3) may merely reflect the fact that maximal mucosal content of folate is achieved on the 8 mg/kg diet (Fig 1). Nevertheless, a non-significant trend towards increased macroscopic neoplasms in the 40 mg/kg group is evident in Figures 2 and 3 and may indicate a difference in the inhibition of carcinogenesis between the 8 and 40 mg/kg groups. A definitive explanation for this trend is not available from our observations, but it should be interpreted in light of the potent procarcinogenic milieu that was used in this study. Supplemental folate may have two distinct actions in this setting: at lower levels of supplementation it seems to possess an inhibitory effect on genesis of microscopic foci of neoplasia, as has been previously described,\textsuperscript{26} as well as the evolution of macroscopic neoplasms from microscopic ones. Independently, in a strongly procarcinogenic environment where the appearance of macroscopic neoplasms is inevitable, exceptionally high supplemental levels may promote the growth of microscopic neoplasms: an ‘acceleration phenomenon’, which has previously been described in humans who have well established cancers and who are given exceptionally large doses of folate.\textsuperscript{39} Studies incorporating larger numbers of animals are necessary to fully determine whether the upward trend between 8 and 40 mg/kg reflects a true difference.

The fact that folate insufficiency increases the risk of, and folate supplementation conveys protection against, colorectal neoplasia remain unclear at present. Because of the intimate relation of folate to biological methylation reactions\textsuperscript{23} and the role of aberrant patterns of DNA methylation in carcinogenesis,\textsuperscript{44} several investigators have proposed that one such mechanism might be altered DNA methylation as a result of change in folate status.\textsuperscript{17, 18, 36} However, the genomic DNA methylation data from our study indicate that moderate folate depletion, and supplementation up to 20 times the daily requirement, in conjunction with DMH treatment, were not associated with changes in genomic DNA methylation. These data suggest that the modulatory effect of dietary folate on colonic neoplasia in the DMH model is not mediated by changes in genomic DNA methylation. This is consistent with recent observations that indicate that a moderate degree of folate depletion, equivalent to that of the rats fed 0 mg folate/kg diet in the present study, does not induce genome-wide or c-myc protooncogene specific DNA hypomethylation in the colon.\textsuperscript{39}

There are several possible explanations why alterations in dietary folate were not observed to alter genomic DNA methylation in the colon. Firstly, the colonic mucosal SAM concentration and the ratio of SAM to SAH, two critical determinants in biological methylation\textsuperscript{23, 45} were similar among the four groups of rats. This may be due to the fact that modulation of SAM and SAH in some tissues such as the colonic mucosa might be particularly resistant to the effect of folate deficiency or supplementation, as indicated by a previous study.\textsuperscript{36} Furthermore, it is known that DMH treatment increases colonic SAM and SAM:SAH ratio in rats.\textsuperscript{51} Therefore, in this study, the effect of DMH on colonic SAM and SAH might have hidden the effect conveyed by alterations in folate status. Another possible explanation for the lack of change in genomic DNA methylation status in this study relates to a direct effect of DMH on the assay utilised to assess DNA methylation. Agents that form adducts at the guanine residue in CpG dinucleotide sequences, such as DMH, can interfere with the binding of DNA methylase to CpG sequences.\textsuperscript{52-55} Therefore, DMH induced adduct formation at the guanine residue in CpG may render these sites resistant to DNA methylation by the Sss1 methylase utilised for assay in this study; the effect of either folate depletion or supplementation might have been masked as a result. However, consistent with human data,\textsuperscript{24, 27} the rats harbouring macroscopic tumours had a significantly lower degree of genomic DNA methylation compared with those without.
Regardless of its explanation, the lack of altered genomic DNA methylation implies that other mechanisms are responsible for mediating the modulatory effect of dietary folate. Recently, two other mechanisms by which folate can modulate colorectal carcinogenesis have been proposed. Folate is an essential factor in the de novo biosynthesis of purines and thymidylate and it is in this manner that folate plays a key part in DNA replication and cell division. Therefore, folate deficiency could contribute to DNA damage and impaired DNA repair, both of which are considered to be important in carcinogenesis. A deficiency of folate in a cell culture system has been observed to potentiate genomic DNA strand breaks induced by DNA damaging agents. Sustained folate deficiency has recently been observed to induce DNA strand breaks both at the genomic level and within a highly conserved area (exons 5 through 8) of the p53 tumour suppressor gene in rat liver. Because DNA strand breaks are associated with neoplastic transformation and the p53 gene is the most frequently implicated gene in colorectal carcinogenesis, genomic or gene specific DNA strand breaks, or both, may be a mechanism by which folate insufficiency increases colorectal carcinogenesis. Folate deficiency has also been shown to cause an imbalance in deoxynucleotide pools; reduction in the deoxythymidylate pool as a result of folate deficiency leads to expansion of the deoxyuridylate pool and to misincorporation of uracil into newly synthesised DNA. Such misincorporation results in an abnormal DNA replication and repair. Furthermore, preliminary experiments indicate that folate deficiency impairs DNA excision repair as well as mismatch repair in the rat colon. Although these mechanisms have not yet been shown to be operative in the folate mediated modulation of colorectal carcinogenesis in the DMH rodent model, the aforementioned candidate mechanisms provide biologically sound explanations for a causal relation between diminished folate status and colorectal cancer and for the protective effect of folate supplementation on colorectal carcinogenesis.

The manner in which the colons were processed in this study precluded histological analysis of the macroscopic lesions. Nevertheless, there is a compelling body of evidence in rodent models of colon cancer, utilising either DMH or its metabolites, which indicates that macroscopic lesions invariably contain neoplastic foci. A deliberate choice was made at the outset of this study to include only those macroscopic lesions with features of neoplasia as described in the literature, non-specific submucosal thickening without vascular abnormalities were specifically excluded from the macroscopic tumour group. Of the 324 macroscopic neoplastic lesions were located in the distal colon, consistent with other studies, whereas almost all submucosal thickenings were found in the proximal colon near the caecum.

In summary, these data corroborate earlier observations that raised folate status may play an inhibitory part in DMH induced colorectal carcinogenesis. These data indicate that the progression from microscopic to macroscopic neoplasms is inhibited by dietary folate. These data also indicate that this modulatory effect extends over a wide range of folate status. Nevertheless, at levels of supplementation exceeding that required for a protective effect was apparent. The implications for chemoprevention in humans are provocative, but unclear at the present time.

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Dietary folate protects against the development of macroscopic colonic neoplasia in a dose responsive manner in rats.

Y I Kim, R N Salomon, F Graeme-Cook, S W Choi, D E Smith, G E Dallal and J B Mason

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