Review

Folate and colonic carcinogenesis: searching for a mechanistic understanding

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A growing body of epidemiologic data indicates a relationship between folate status and the risk of developing colorectal neoplasia. Diminished folate status is associated with a higher risk of neoplasia. Interestingly, this phenomenon has been observed with relatively modest alterations in folate status and may even be present when vitamin status is diminished within the range of what is conventionally accepted as normal. A controlled laboratory study in an animal model of colorectal carcinogenesis confirmed a cause and effect relationship between folate deficiency and enhanced carcinogenesis. Possible means by which this effect may occur are discussed. Preliminary studies in humans with adenomatous polyps or colorectal cancers suggest that an intermediary marker of colonic tumorigenesis may be reversed by administering pharmacologic doses of folic acid, indicating a possible chemopreventive role for this vitamin. (J. Nutr. Biochem. 5:170–175, 1994.)

Keywords: folate; colon cancer; carcinogenesis; cancer chemoprevention

Introduction

A provocative array of observations from both laboratory and clinical investigations indicates that alterations in folate status may modulate the process of neoplastic transformation in the colon. Diminished folate status appears to promote colonic carcinogenesis. Considerably more speculative is the concept that supraphysiologic folate status might afford some protective effect. This discussion will review these observations and outline some of the possible mechanisms by which these effects may be exerted.

An interest in folate and cancer dates back to the 1960s when Van Niekerk first noted several cytologic similarities between epithelial cells of the uterine cervix from folate-deficient women ("megaloblastic cells") and those cervical cells that are undergoing the early stages of neoplastic transformation and are therefore considered to be precancerous ("dysplastic cells").[1] In both cases, the cells are larger than normal, have nuclei that are disproportionately large compared with the size of their cytoplasm, and the chromatin is hyperchromatic. Initially, these similarities were explained as an interesting cytologic coincidence. However, in the 1970s and 1980s, investigators began to question whether there might be a functional association between megaloblastic and dysplastic cells. Two prospective, controlled clinical intervention trials, conducted in the 1980s, studied individuals with dysplastic, or metaplastic, changes in the epithelia of the uterine cervix[2] and bronchus.[3] Each of these studies observed a significant degree of attenuation or regression in dysplasia after several months of supplementation with pharmacologic doses of folic acid alone[2] or in conjunction with pharmacologic doses of vitamin B_{12}.[3] This lent further support to the concept that folate and dysplasia might be functionally related. A subsequent clinical trial, conducted in a fashion that more closely controlled for confounding variables, substantiated an association between diminished folate status and the risk of dysplasia in the uterine cervix,[4] although a more recent intervention trial in subjects with cervical dysplasia[5] was not able to reproduce the attenuation of dysplasia noted by the earlier study.

Clinical observations from several different groups also point to an association between diminished folate status, assessed by dietary intake of the nutrient or by measurement

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of blood levels, and an enhanced risk of colorectal adenomas or cancer. Lashner et al. first made this observation in a large retrospective, case-control study of individuals afflicted with chronic ulcerative colitis,[6] a disease that not only carries with it a 10- to 40-fold increased risk of colonic cancer but a significant risk of folate deficiency.[7,8] The authors determined that individuals who had not been taking folate supplements over the long term had a rate of colonic neoplasia that was approximately 2.5-fold greater than those who had been taking supplements. Furthermore, chronic administration of sulfasalazine, a drug commonly used for the treatment of ulcerative colitis and one that is well known to specifically inhibit both folate absorption[8,9] and folate metabolism,[10] was associated with a 50% increase in the risk of dysplasia. Both of these observations fell slightly short of conventionally accepted levels of statistical significance but the study, nevertheless, established the importance of examining this issue in the colon. More recently, Lashner confirmed these observations by prospectively assessing erythrocyte (RBC) folate levels in ulcerative colitis patients who harbor neoplastic changes in their colorectum with age-, sex-, and disease-matched controls who do not harbor any neoplastic lesions:[11] this analysis demonstrated that the RBC folate level was 66.2 ng/mL lower in the individuals with dysplasia or cancer than in the controls. The group harboring neoplastic lesions consisted of four subjects with dysplasia and two with cancer. No evidence of general malnutrition was observed among these individuals and therefore the lower mean folate level in this group was not felt to be a result of the underlying neoplasia. An 18% incremental increase in the relative risk of colorectal neoplasia was observed for every drop of 10 ng/mL in the RBC folate. It is noteworthy that although the group that harbored neoplasia had diminished RBC folate levels compared with the control group, the absolute level in the neoplasia group (454 ng/mL) was well within the range of values that is conventionally accepted as normal.

Epidemiologic studies of the general population: one based in western New York State (USA)[12] and the other on the island of Majorca,[13] also observed that the larger the intake of dietary folate, the lower the rate of colorectal cancer, in the case of the Majorca study, or for rectal cancer alone, in the case of the New York study. The most convincing epidemiologic evidence to date to establish an association between folate status and colorectal neoplasia was recently published by Giovannucci et al.[14] The prospective design of this study, the large population included in the analysis (n = 25,474), and the control of several confounding dietary variables enabled these investigators to convincingly support the hypothesis that increased folate intake is inversely associated with the incidence of adenomatous polyps in the distal half of the colorectum. These studies in the general population are particularly important because they demonstrate an apparent effect in populations that do not clearly have other, predisposing, conditions to colorectal cancer.

Establishing a causative relationship between folate status and colonic carcinogenesis

However provocative these clinical studies might be, they can only establish an associative, and not causative, relationship. Two of the three intervention studies[2,3] imply a causative relationship between folate deficiency and cancer, although the reversal of dysplasia with folate supplementation is a concept that does not necessarily imply that diminished folate status promotes the process of carcinogenesis. Furthermore, some of the apparent regression of dysplasia observed in the intervention trials might, in fact, represent reversal of megaloblastic changes in the cells that masqueraded as dysplasia.

Our laboratory therefore set out to conduct a study to more clearly establish whether a cause-and-effect relationship exists between diminished folate status and colonic carcinogenesis. The dimethylhydrazine rat model of colonic carcinogenesis, which has been extensively studied,[15] was chosen to examine this issue. Weekly subcutaneous injections of 1,2-dimethylhydrazine dihydrochloride, a procarcinogen whose metabolites are thought to exert their carcinogenic effect by alkylation of DNA, produces a sequence of hyperplasia, followed by increasing grades of dysplasia, and finally cancer in the colonic mucosa over a period of several months. Histologically, these events are similar to those that are thought to occur in human colonic carcinogenesis, although the time course is greatly accelerated. Many of the genomic events commonly observed in human colonic carcinogenesis, such as c-*myc* proto-oncogene overexpression and k-*ras* activation, are also observed in this model.[16,17] Folate-replete rats were fed an amino acid-defined diet containing 8 mg folic acid/kg of chow; the deplete group received an identical diet except it contained 0 mg/kg.[18] Neither group received succinylsulfathiazole, a non-absorbable antibiotic typically added to produce a more marked degree of folate deficiency. Severe deficiency was therefore avoided because of the endogenous synthesis of folate by intestinal bacteria and copraphagy.[19] The degree of folate deficiency that was created in the deficient groups was therefore moderate enough to maintain good health in the animals and to prevent any difference in the growth curves between the deficient and replete animals.[20] After 5 weeks of carcinogen exposure, and at 5 week intervals thereafter, rats from each of the groups were killed; tissue samples of blood, liver, and colonic mucosa were harvested for determination of folate concentrations; and the colorectum from each animal was fixed and stained for conventional histologic examination. Two pathologists, blinded to the groupings of the rats, independently examined identical representative longitudinal sections of colorectum from each animal and the total number of dysplastic and cancerous foci were tallied. *Figure 1* demonstrates that the folate-deficient rats with the underlying predisposition to colonic neoplasia started developing colonic neoplasia at a greater rate by 15 weeks and by 20 weeks had a significantly greater incidence of neoplasia than folate-replete controls.[20] No cancer was observed in the two control groups that received saline injections in conjunction with either a folate-replete or deplete diet.

In this rat model of colonic carcinogenesis, moderate folate deficiency enhances the development of colonic dysplasia and cancer, thereby providing considerable evidence to a cause-and-effect relationship between diminished folate status and colonic cancer.

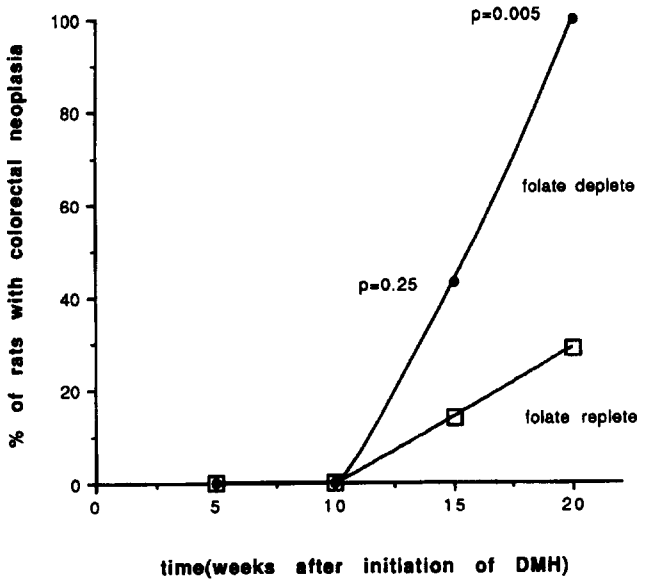

Figure 1 Incidence of colonic dysplasia and/or carcinoma in folate-deplete (closed circles) and folate-replete (open squares) rats that had been treated with the procarcinogen, dimethylhydrazine (DMH). At 20 weeks there was a significant difference between the two groups. Adapted from Cravo et al. with permission.[20]

Diminished folate status and enhanced colonic carcinogenesis: potential mechanisms

The mechanism by which diminished folate status acts to enhance colonic carcinogenesis is not known. By necessity, therefore, any discussion pertaining to the mechanism must be speculative. There are many potential mechanisms by which this effect may occur, and there is no a priori reason to believe that multiple mechanisms might not play a role *(Table 1)*. Because all the known biochemical functions of folate pertain to its ability to mediate the transfer of one-carbon fragments,[21] it is possible that one or more of the mechanisms that have been hypothesized to mediate other examples of lipotrope deficiency-associated carcinogenesis may also be responsible for enhanced carcinogenesis associated with the isolated depletion of folate.

Diminished folate status may have some influence on the patterns of DNA methylation and thereby might influence gene expression. Mammalian DNA is methylated at deoxycytidine residues; methylation of the other nucleotides probably does not occur but has not been rigorously excluded.[22] Virtually all of these methylated residues reside in cytosine-guanine (5′-CpG-3′) sequences; a dinucleotide sequence that is disproportionately clustered in "CpG-rich islands" located at the 5′ end of genes.[23] Methyltetrahydrofolate provides the methyl group for methionine, and therefore S-adenosylmethionine (SAM), synthesis.[24,25] Because SAM is the proximal methyl donor for DNA methylation[26] *(Figure 2)*, it is not surprising that rats who are fed diets devoid of methionine, choline, B_{12}, and folate have been observed to have low hepatic SAM concentrations and diminished hepatic DNA methylation.[27] Rats that are fed diets lacking choline, or lacking choline and other lipotropes, have marked enhancement of chemically induced hepatocarcinogenesis;[28] when such diets are prolonged, the animals appear to spontaneously develop these cancers.[29] Preceding the development of the cancers is the appearance of global DNA hypomethylation,[27] specific hypomethylation of the c-*myc*, c-*fos*, and c-Ha-*ras* proto-oncogenes and elevated steady-state levels of the corresponding mRNAs.[30] All three of these proto-oncogenes have been associated with hepatocarcinogenesis in the rodent. These observations are consistent with a considerable body of literature that indicates that altering the methylation of certain genes is an important means of regulating gene expression,[31] probably by modulating the interaction between the promotor sites of genes and the transcription machinery.[32] In most instances, hypomethylation of the 5′ end of genes, which are regulated by this phenomenon, is associated with enhanced transcription. Further evidence supporting a mechanistic role for DNA hypomethylation in colonic carcinogenesisis is provided by observations that indicate that global DNA hypomethylation is almost invariably observed in early neoplastic lesions of the colorectum in humans, often before other genomic events that have been associated with colon carcinogenesis appear.[33,34] In some cases, hypomethylation of certain colon cancer-associated proto-oncogenes such as k-*ras* have also been observed.[35] Aberrant DNA hypomethylation has also been proposed as the mechanism by which genomic imprinting, the heritable

Table 1 Candidate mechanisms for folate-related enhancement of colorectal carcinogenesis

- Induction of DNA hypomethylation
- Secondary deficiency of choline
- Diminution in natural killer cell surveillance
- Increased chromosomal fragility
- Misincorporation of uridylate for thymidylate in DNA synthesis

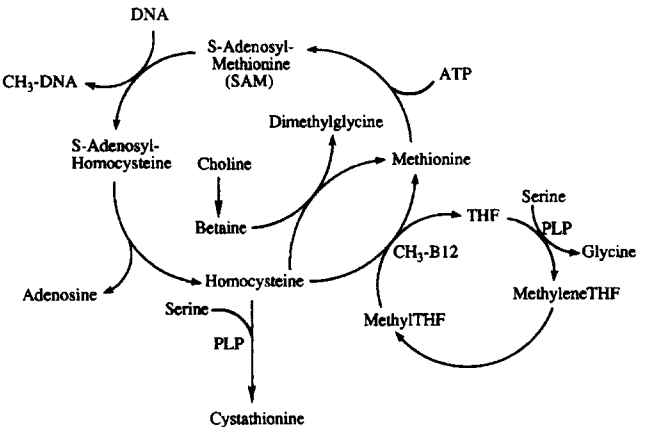

Figure 2 The sulfur-containing amino acid pathway. 5-methyltetrahydrofolate, located in the lower righthand corner of this diagram, is a substrate that supplies the methyl group for methionine synthesis. Methionine is then adenylated to produce S-adenosylmethionine. This compound is the proximal methyl donor for DNA methylation. Alternatively, methionine can be synthesized from betaine, which is an oxidation product of choline. Abbreviations: PLP, pyridoxal 5-phosphate; THF, tetrahydrofolate. Adapted from Selhub, J. and Miller, J. (1992). The pathogenesis of homocysteinemia. *Am J Clin Nutr* **55,** 131–138.

suppression of either the maternal or paternal allele, is lost in human cancer.[36,37] This provides another avenue by which folate deficiency might predispose to inappropriate gene expression. Nevertheless, this is an increasingly complex field, because site-specific DNA hypermethylation has also been observed in colonic carcinogenesis, including sites that contain important tumor suppressor genes such as p53.[38,39]

Whether diminished folate status alone, a nutritional manipulation known to decrease SAM levels,[24,25] may induce global DNA hypomethylation is still a matter of debate. One recent report suggests that severe folate deficiency in rats produces global DNA hypomethylation,[40] but this observation needs to be confirmed. Whether gene-specific hypomethylation is present in folate deficiency is under active investigation.

Another mechanism that might play a role in the procarcinogenic effects of diminished folate status is by inducing a secondary depletion of choline. Choline deficiency in animals is known to serve as a promotor of hepatocarcinogenesis and prolonged choline deficiency is, by itself, a sufficient condition to produce hepatocellular carcinomas.[41,42] Colonic neoplasia has not clearly been shown to be a consequence of choline deficiency although, in the case of the colon, an initiator or other underlying predisposition may need to be present for the choline deficiency to result in colonic cancer. The mechanism by which choline deficiency mediates these effects is not known, but it has been suggested that the increased intracellular 2,3 diacylglycerol levels observed in choline deficiency is of sufficient magnitude to activate protein kinase C (PKC) and induce its shift from a cytosolic to cell membrane compartment.[43] Activation of protein kinase C is associated with mitogenesis and enhanced expression of the c-*myc* proto-oncogene;[44,45] furthermore, fibroblasts transfected with a mutant form of PKC that is continuously activated, acquire a neoplastic phenotype.[46] Activation of PKC is also apparent in colonic carcinogenesis: it has been shown to occur early in the development of chemically induced colonic neoplasia[47] as well as in human colorectal cancers.[48,49] To understand how folate deficiency might promote carcinogenesis by the same pathway it is necessary to recall some in vitro observations of Finkelstein et al., which indicate that impairing methionine synthesis in rat liver homogenates by removal of methylfolate leads to enhanced activity of the alternative pathway for methionine synthesis, the transmethylation of homocysteine by betaine.[50] Betaine, in turn, is obtained from the oxidation of choline *(Figure 2)*. A long-term deficiency of folate might, therefore, lead to increased utilization of betaine and choline in the liver and to a significant enough depletion of choline in the liver and peripheral tissues to increase 2,3 diacylglycerol levels and to activate protein kinase C. This is a hypothesis that has yet to be proven.

Folate deficiency is known to have adverse effects on the integrity of lymphocyte function, as assessed by the blastogenic response to mitogens.[51] Natural killer cells, a subset of lymphocytes, are felt to be responsible for the surveillance and destruction of arising clones of neoplastic cells.[52] A rodent study demonstrated that severe folate deficiency will suppress the ability of lymphocytes to kill heterologous cells to which the rodents had previously been sensitized.[53] One could hypothesize, therefore, that diminished folate status might impair the ability of natural killer cells to destroy dysplastic or cancerous cells.

Cancers in many tissues, including the colon, have been linked to disruptions in chromosomal integrity; often leading to the loss of tumor suppressor gene activity.[39] The induction of folate deficiency in cultured lymphocytes is known to induce a variety of such chromosomal abnormalities at foci called folate-sensitive "fragile sites," some of which are closely linked to known proto-oncogenes and anti-oncogenes.[54,55] There are also observations that suggest that folate deficiency may produce such an effect in vivo.[56]

The last mechanism mentioned in *Table 1* pertains to the creation of imperfect DNA copies during cell division due to the misincorporation of deoxyuridylate for thymidylate in the setting of folate deficiency. When human lymphoid cells are treated with potent inhibitors of folate metabolism, such as methotrexate, de novo thymidine synthesis, which is folate-dependent, drops to low enough levels that uridylate is incorrectly substituted in newly synthesized DNA where thymidylate should be.[57] The implications of this phenomenon as it pertains to cellular metabolism are not yet defined, but it suggests a means by which impaired folate status could play a role in the creation of mis-sense or non-sense mutations.

Although tumorigenic viruses are not felt to play a significant role in colorectal carcinogenesis, these viruses may play a very important role in the development of other types of cancer, such as those of the uterine cervix. Diminished folate status may promote the development of such cancers by interacting with the biology of these viruses. The human papilloma virus-18 (HPV-18), which is highly associated with human cervical neoplasia, specifically incorporates into the human genome adjacent to four loci, three of which are in or near a constitutive fragile site.[58] Diminished folate status in vitro[54,55] and, perhaps, in vivo[56] is known to distort chromosomal architecture at such sites and thereby might facilitate incorporation of the viruses.

In summary, clinical observations, as well as a controlled laboratory study in an animal model, indicate that diminished folate status does enhance colonic carcinogenesis. A great deal of work remains, however, to understand the means by which this occurs.

Can augmentation of folate status reduce the risk of colonic carcinogenesis?

Alterations in DNA methylation are commonly observed as an early biochemical phenomenon in colonic carcinogenesis.[33–35,38,39] Global hypomethylation of DNA has been observed frequently in early, pre-malignant, colonic neoplasias,[33,34] and hypomethylation of certain proto-oncogenes, such as k-*ras,* has been documented to occur.[35] Therefore, in addition to serving as a possible mechanism by which folate deficiency enhances carcinogenesis, DNA hypomethylation may prove to be a satisfactory intermediary marker of colonic carcinogenesis. Interestingly, DNA hypomethylation precedes most of the other known molecular events that occur in colorectal tumorigenesis,[59] suggesting that alterations in DNA methylation may play an active role in the evolution of carcinogenesis. Therefore, it is of interest to

determine whether the DNA hypomethylation associated with early dysplasia can be modified by intervention. Although the DNA hypomethylation observed in early adenomatous polyps in humans is not likely a consequence of folate deficiency, it is of considerable interest that preliminary clinical studies in individuals who have either colorectal adenomas or cancers indicate that pharmacologic doses of folic acid reverse the global DNA hypomethylation that is present in the colonic epithelium.[60] This has important implications for determining the mechanism by which folate status modulates colonic carcinogenesis and has a potential role in the prevention of colorectal cancer.

Conclusions

The balance of present evidence indicates that folate status can modulate the process of colonic carcinogenesis. Interestingly, these effects seem to occur with relatively modest alterations in folate status, and may even be present when vitamin status is altered within the range of what is presently considered to be normal. Folate, as a compound whose only known functions relate to its transfer of one-carbon units, therefore appears to have joined the other lipotropes as a compound that might modulate carcinogenesis. The mechanisms by which this may occur are diverse and are under intense investigation.

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