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Role of Folate in Colon Cancer Development and Progression[1]

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ABSTRACT Folate, a water-soluble B vitamin and important cofactor in 1-carbon transfer, is an important nutritional factor that may modulate the development of colorectal cancer. Epidemiologic and clinical studies indicate that dietary folate intake and blood folate levels are inversely associated with colorectal cancer risk. Collectively, these studies suggest an ~40% reduction in the risk of colorectal cancer in individuals with the highest dietary folate intake compared with those with the lowest intake. Animal studies using chemical and genetically predisposed rodent models have provided considerable support for a causal relationship between folate depletion and colorectal carcinogenesis as well as a dose-dependent protective effect of folate supplementation. However, animal studies have also shown that the dose and timing of folate intervention are critical in providing safe and effective chemoprevention; exceptionally high supplemental folate levels and folate intervention after microscopic neoplastic foci are established in the colorectal mucosa promote rather than suppress colorectal carcinogenesis. These animal studies in conjunction with clinical observations suggest that folate possesses the dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention. Folate deficiency has an inhibitory effect whereas folate supplementation has a promoting effect on progression of established neoplasms. In contrast, folate deficiency in normal epithelial tissues appears to predispose them to neoplastic transformation, and modest levels of folate supplementation suppress the development of tumors in normal tissues. Notwithstanding the limitations associated with animal models, these animal studies suggest that the optimal timing and dose of folate intervention need to be established for safe and effective chemoprevention in humans. J. Nutr. 133: 3731S–3739S, 2003.

KEY WORDS: • *folate* • *colorectal cancer* • *chemoprevention* • *animal models*

Folate, a water-soluble B vitamin, recently emerged as an important nutritional factor that appears to play an important role in the pathogenesis of several disorders in humans including macrocytic anemia, cardiovascular disease (1,2), thromboembolitic processes (3), neural tube and other congenital defects (4,5), adverse pregnancy outcomes (6,7), and neuropsychiatric disorders (8). The expanding role of folate nutrition in health and disease has major public health implications. For example, evidence for a protective effect of folate supplementation on neural tube defects (4,5) was considered to be sufficiently conclusive and led the U.S. Food and Drug Administration to issue a regulation in 1996, to be effective by January 1998, requiring that all flour and uncooked cereal grain products in the United States be fortified with folic acid (140 μg/100 g) (9). Mandatory fortification was also implemented in Canada (10). An accumulating body of evidence over the past decade suggests that folate may also play a significant role in the development and prevention of several malignancies including cancer of the colorectum, lungs, pancreas, esophagus, stomach, cervix, and breast and neuroblastoma and leukemia (11,12). These studies collectively suggest an inverse association (in some cases dose dependent) between folate status, either folate intake (dietary and supplemental) or blood measurements of folate, and the risk of these malignancies (11,12). The precise nature and magnitude of the inverse relationship between folate status and the risk of these malignancies, however, have not been clearly established (11,12). Thus, the potential role of folate as a cancer preventive agent still remains one of the most speculative and provocative new medical applications of folate nutrition (13).

Epidemiologic evidence for the role of folate in colorectal carcinogenesis

The role of folate in carcinogenesis has been best studied for colorectal cancer (12,14,15). Among 15 published retrospectively conducted epidemiologic studies that investigated the relationship between folate status (assessed by dietary folate intake or by the measurement of blood folate levels) and

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the risk of colorectal cancer or its precursor, adenoma, the majority showed either a significant or equivocal inverse relationship that was not statistically significant, that became nonsignificant after adjustment, or that could not be distinguished from other factors in their relation to risk (12,14,15). Some of these studies demonstrated site (i.e., colon versus rectum) and sex specificity and a dose-dependent association (12,14,15). The relationship between blood levels of folate and the risk of colorectal cancer and adenoma is less well defined than that between dietary intake and the risk of colorectal neoplasms (12,14,15). Collectively, these retrospective studies suggest an ~40% reduction in the risk of colorectal neoplasms in subjects with the highest dietary folate intake compared with those with the lowest intake (12,14,15). These studies also suggest that a modest reduction in folate status without overt clinical evidence of folate deficiency is sufficient to enhance colorectal cancer (12,14,15). A recent meta-analysis of 11 prospective epidemiologic studies from the United States, Canada, The Netherlands, and Sweden including over 500,000 male and female subjects demonstrated a significant inverse association between folate intake (dietary and supplemental) and the risk of colorectal cancer (David Hunter, presented at the 2003 Environmental Mutagen Society Colon Cancer Conference, Miami Beach, FL, May 14–16, 2003). This meta-analysis also showed a 20% reduction in the risk of colorectal cancer in subjects with the highest folate intake compared with those with the lowest intake. One of the most convincing pieces of evidence comes from a prospective study involving 88,756 female nurses in the Unites States (the Nurses' Health Study), which has shown a 75% reduction in colorectal cancer risk in women using multivitamin supplements containing ≥400 μg of folic acid for ≥15 y compared with those not taking folic acid after all the known confounding factors were controlled for.

In some epidemiologic studies, the observed inverse association between folate status and colorectal cancer risk was further modified by the intake of alcohol, a known folate antagonist (16), and other methyl group donors (e.g., methionine, vitamins B-6 and B-12) that are involved in the folate metabolic pathway (12,14,15). Recent molecular epidemiologic studies also showed that the C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR)[3] gene may modulate colorectal cancer risk and that the direction and magnitude of the risk modification are influenced by folate status, alcohol consumption, and the supply of methyl group donors (17–20). MTHFR is a critical enzyme in folate metabolism that catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, thereby playing an important role in DNA synthesis, maintenance of nucleotide pool balance, and DNA methylation (**Fig. 1**) (12,21). The MTFHR C677T polymorphism causes thermolability and reduced MTHFR activity leading to lower levels of 5-methyltetrahydrofolate, an accumulation of 5,10-methylenetetrahydrofolate, increased plasma homocysteine levels (a sensitive inverse indicator of folate status), changes in cellular composition of 1-carbon folate derivatives, and DNA hypomethylation (Fig. 1) (12,21). These studies collectively provide a paradigm of nutrient-nutrient and gene-nutrient interactions in colorectal carcinogenesis, an emerging important topic in the field of nutrition and cancer (21).

Folate also appears to play a role in colorectal carcinogenesis associated with ulcerative colitis. Chronic ulcerative colitis is associated with a 10–40-fold increased risk of developing

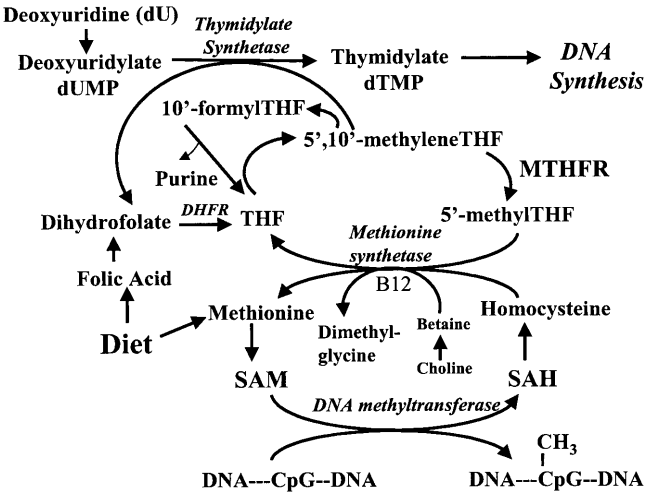

FIGURE 1 Simplified scheme of folate involving DNA synthesis and methylation. B12, vitamin B-12; DHFR, dihydrofolate reductase; CH_3, methyl group; CpG, cytosine-guanine dinucleotide sequence; MTHFR, methylenetetrahydrofolate reductase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate.

colorectal cancer compared with the general population (22). A recent meta-analysis of all published studies reporting a colorectal cancer risk in ulcerative colitis since 1925 reported the risk for patients with ulcerative colitis to be 2% after 10 y, 8% after 20 y, and 18% after 30 y of disease (23). Although megaloblastic anemia is rare, patients with ulcerative colitis often demonstrate depressed blood concentrations of folate due to the frequent use of sulfasalazine, a known folate antagonist, inadequate nutritional intake, and intestinal losses from inflammation (12). Lashner et al. (24) reported that individuals with longstanding ulcerative colitis taking folate supplementation had a nonsignificant 62% lower incidence of colorectal dysplasia and cancer compared with those not receiving folate supplementation [odds ratio (OR), 0.38; 95% confidence interval (CI), 0.12–1.20]. In another study the risk of colorectal dysplasia and cancer was found to be significantly decreased by 18% for each increase of 23 nmol/L (10 ng/mL) in red blood cell folate concentrations in patients with ulcerative colitis (OR, 0.82; 95% CI, 0.68–0.99) (25). Folic acid supplementation was inversely related to the risk of colorectal neoplasia in subjects with longstanding ulcerative colitis in a dose-dependent manner (relative risk, 0.54 and 0.76 for 1.0 and 0.4 mg of folic acid/d, respectively) (26). Although these studies had small sample sizes and the inherent limitations associated with retrospective study designs, they nevertheless suggest a provocative inverse relationship between folate status and the risk of colorectal cancer associated with ulcerative colitis.

In summary, a growing body of observational epidemiologic studies has suggested that folate deficiency increases whereas folate supplementation decreases the risk of colorectal cancer. Although the results from these studies are not uniformly consistent, the portfolio of evidence strongly supports the inverse association between folate status and colorectal cancer risk. However, these observational epidemiologic studies only suggest this association and cannot definitively establish a causal relationship between folate status and colorectal cancer risk.

Folate intervention studies in humans

In theory, randomized intervention studies in humans should provide definitive support for the purported cause-and-effect

[3] Abbreviations used: AOM, azoxymethane; DMH, dimethylhydrazine; MNU, *N*-methyl-*N*-nitrosourea; MTHFR, methylenetetrahydrofolate reductase; SEPB, surrogate end point biomarkers.

relationship between folate status and colorectal cancer. However, it was recently suggested that intervention studies should not be considered as an epidemiologic gold standard in the field of nutrition and cancer (27). Nevertheless, controlled trials in which intervention shows beneficial effects are good evidence that the agents used are protective whereas studies in which intervention shows no effect or even a detrimental effect do not show that the agents used are irrelevant or harmful in the context of whole diets or among normal, healthy populations (27). At present, no conclusive evidence from human experiments supports the protective effect of folate supplementation on colorectal carcinogenesis.

Several small randomized, placebo-controlled intervention studies have examined the effect of folate supplementation on colorectal cancer risk. Cravo et al. (28) observed that folate supplementation at a dose of 10 mg/d for 6 mo in 22 patients with resected colonic adenoma or cancer significantly reversed a purported biomarker of colorectal cancer (genomic DNA hypomethylation) in the normal rectal mucosa. During the washout period, DNA methylation values moved toward the initial values in most cases. In a subsequent study, Cravo et al. (29) observed that 3 mo of folate supplementation (5 mg of folate/d) significantly reversed genomic DNA hypomethylation in the normal rectal mucosa in 20 patients with single, but not multiple, resected colorectal adenomas compared with placebo. Investigators from Greece reported a study involving 60 subjects with colorectal adenoma: folate supplementation (1 mg/d for 2 y) after polypectomy decreased adenoma recurrence by 46% compared with placebo, although this difference was not statistically significant (30). In a pilot study from Boston ($n = 20$), folate supplementation at 5 mg/d for 1 y after removal of adenomas significantly accelerated improvement in genomic DNA methylation and *p53* strand breaks in exons 5–8 in the normal rectal mucosa at 6 mo compared with placebo, but these effects were no longer significantly different at 1 y (31). A recent study from the United Kingdom (32) demonstrated that folate supplementation at 2 mg/d for 3 mo in 11 subjects with previously resected colorectal adenomas significantly decreased rectal mucosal cell proliferation, a purported surrogate end point biomarker (SEPB) of colorectal cancer (33).

Folate chemoprevention trials were also performed in patients with chronic ulcerative colitis at risk of colorectal dysplasia and cancer. In one trial, folinic acid, a reduced and 1-carbon-substituted form of folate, significantly reduced cell proliferation in the upper portion of the colonic crypts (34). In a study done in Portugal, however, folate supplementation at 5 mg/d for 6 mo failed to reverse genomic DNA hypomethylation in patients with chronic ulcerative colitis (35). A case report suggested that folate supplementation at 5 mg/d for 6 mo partially corrects microsatellite instability, the hallmark of DNA mismatch repair defects observed in hereditary, sporadic, and ulcerative colitis–associated colorectal carcinogenesis (36,37), in a patient with chronic ulcerative colitis (38). Lashner et al. (39) showed that folate supplementation may protect against the development of *p53* mutations, a common and early event in ulcerative colitis-associated colorectal carcinogenesis (40), in subjects with chronic ulcerative colitis.

The number of subjects studied in the aforementioned trials is too small, the duration of follow-up was relatively short, and most of these studies used less well-established SEPBs of colorectal cancer instead of adenoma or cancer occurrence or recurrence as the endpoint of the trial. Therefore, it is difficult to draw any definitive conclusions about the chemopreventive role of folate supplementation in colorectal carcinogenesis from these small trials. There are several large, randomized, double-blind, placebo-controlled multicenter folate chemoprevention trials ongoing in the United States (National Cancer Institute communication, 2003). However, even these trials may not be able to provide definitive conclusions about the chemopreventive role of folate supplementation in colorectal carcinogenesis for several reasons. First, these intervention studies attempt to intervene in incompletely understood biological pathways in special populations of adults at high risk of developing colorectal cancer (i.e., individuals with previously resected colorectal adenomas) who therefore may be at a late, although preclinical, stage of colorectal carcinogenesis. Second, the time between the change in the level of folate intake and any expected change in the incidence of colorectal cancer (i.e., relevant induction time) is usually uncertain and trials of 3–5 y may not be sufficiently long for any significant effect associated with folate supplementation to be apparent. Third, subjects who agree to participate in trials tend to be relatively health conscious and highly motivated; individuals who are at high potential risk on the basis of dietary intake, and thus susceptible to intervention, are liable to be underrepresented. Hence, the validity of generalizing the results is limited. Fourth, these trials use SEPBs of colorectal cancer as the outcome instead of using occurrence or recurrence of cancer. All SEPBs have limitations and most have not been conclusively validated in clinical studies (41). Except for a few biomarkers (e.g., adenomas [42]), modulating any of these SEPBs has not yet clearly led to reduction in colorectal cancer incidence and mortality (41). Even for adenomas the risk of progression to adenocarcinoma depends on the histologic type, size, and number and hence all adenomas cannot be considered to possess a similar colorectal cancer risk (42). Therefore, the results from intervention studies should be interpreted with utmost caution and should not be treated as an unequivocal confirmation or refutation of evidence concerning the relationship between folate status and colorectal cancer risk obtained from other types of epidemiologic studies.

Evidence from animal studies: the dual modulatory effects of folate status on colorectal carcinogenesis depending on the timing and dose of folate intervention

The role of folate in colorectal carcinogenesis has been investigated in several animal studies using chemical carcinogens and in genetically predisposed rodent models of colorectal cancer. Collectively, these studies have corroborated the inverse association between folate status and colorectal cancer risk observed in epidemiologic studies. These studies have also shown that the dose and timing of folate intervention are critical in providing safe and effective chemoprevention.

Chemical carcinogen rodent models of colorectal cancer. The first study that unequivocally established the causal association between folate deficiency and colorectal cancer was by Cravo et al. (43). In this study, 100% of male Sprague-Dawley rats fed a folate-deficient diet (0 mg folic acid/kg diet) developed microscopic colorectal neoplasms 20 wk after initiation of dimethylhydrazine (DMH, a well-established colorectal carcinogen) injections compared with 29% of the rats fed a folate-sufficient diet (8 mg of folic acid/kg diet). The incidence of macroscopic colorectal neoplasms was 86% and 43% in the folate-deficient and sufficient groups, respectively ($P = 0.09$). This study suggests that folate deficiency affects an early phase of colorectal carcinogenesis in this rodent model. Another important finding was that no cancer was observed in the two control groups that received saline injections in conjunction with either a folate-deficient or -sufficient diet, thereby suggesting that folate deficiency alone is not sufficient

to initiate carcinogenesis but rather potentiates other risk factors for carcinogenesis. The degree of folate depletion induced in this study was moderate enough to permit maintenance of good health of animals and to prevent growth retardation, anemia, and premature death. This study, therefore, corroborated epidemiologic and clinical observations that mild-to-moderate folate depletion is sufficient to enhance carcinogenesis.

In a subsequent study using the same DMH rodent model of colorectal cancer, the same folate-deficient diet (0 mg of folic acid/kg diet) was associated with a potentiation of the development of macroscopic colorectal tumors (44). Dietary folate supplementation up to 4 times (8 mg of folic acid/kg diet) the basal dietary requirement (2 mg of folic acid/kg diet) was shown to retard the progression from microscopic colorectal neoplastic foci to macroscopic tumors in a dose-dependent manner. This study suggests that folate also has a modulatory effect in a later stage of colorectal carcinogenesis. Levels of dietary folate >4 times the basal dietary requirement did not convey additional benefits; in fact, there was a nonsignificant trend toward increased colorectal tumorigenesis in rats fed a supraphysiological dose of folate (20 times the daily requirement; 40 mg of folic acid/kg diet). This study suggests that supplemental folate may have two distinct actions in this model. At modest levels of supplementation beyond the dietary requirement, folate seems to possess an inhibitory effect on the genesis of microscopic neoplastic foci as well as the progression of macroscopic neoplasms from microscopic foci. In a strongly procarcinogenic environment where the appearance of microscopic neoplasms is inevitable, however, exceptionally high supplemental folate levels may promote the progression of chemically induced colorectal neoplastic foci. In support of this latter finding, dietary folate supplementation exceeding the basal requirement by 1000 times (2.0–5.0 g of folic acid/kg diet) increased the development of aberrant crypt foci, the probable earliest precursor of colorectal cancer (45), compared with a control diet (AIN-76A; 2 mg of folic acid/kg diet) in other animal studies using a different strain of rats (Fischer 344) and another colorectal carcinogen, azoxymethane (AOM, a metabolite of DMH) (46–48).

Two recently published studies using male Sprague-Dawley rats and AOM suggested that contrary to the previously observed enhancing effect of folate deficiency on colorectal carcinogenesis, folate deficiency in fact reduces the development of colorectal cancer in rats (49,50). One study (49) demonstrated that a folate-deficient diet (0 mg of folic acid/kg diet) significantly reduced the occurrence of aberrant crypt foci compared with the control diet (8 mg of folic acid/kg diet) whereas the other study (50) showed that the same folate-deficient diet (0 mg of folic acid/kg diet) significantly decreased the incidence and multiplicity of tumors in the small and large intestine compared with the control diet (8 mg of folic acid/kg diet). The latter study, however, showed that the protective effect of folate deficiency was only on tumors in the small intestine and not on colonic tumors (50). When the colonic tumors were further classified into adenomas and adenocarcinomas, the folate-deficient diet was shown to reduce the multiplicity of colonic adenocarcinomas but not adenomas compared with the control diet (50). Careful inspection of the protocol used in these studies provides a ready explanation for the apparent inconsistency in results. In fact, these studies are complimentary and demonstrate similar principles in regard to folate's role in the modulation of colorectal carcinogenesis.

The study design and diets used in these studies (49,50) are significantly different from those used in previous studies (43,44) that unequivocally established the potentiating effect of folate deficiency on colorectal cancer development in several aspects. First, these studies (49,50) used the casein-based AIN-93 semipurified diet instead of the amino acid–defined diet used in the previous studies (43,44). The amino acid–defined diets constitute a standard method of predictably inducing folate deficiency or providing supplemental dietary folate in rodents (51) whereas casein-based diets contain measurable levels of folate and hence cannot predictably modulate folate levels in rodents in a consistent fashion. Second, the casein-based diet used in these studies (49,50) contained a twofold higher content of fat compared with the amino acid–defined diet (20% versus 10% by weight). High-fat diets have been shown to promote colorectal carcinogenesis in humans and animals (52) and hence might have confounded the effect of dietary folate. Third, the colonic folate concentrations reported for the deficient groups in these studies (49,50) greatly exceed (by 10,000-fold) levels normally expected for deficient rodents as observed in the previous studies (43,44) (3467 μg/g vs. 0.17–0. 48 μg/g). Even the colonic folate concentrations in the control groups in these studies exceed by 10,000-fold the usual levels achieved by 8 mg of folic acid/kg diet as observed in the previous studies (8800 μg/g vs. 0.56–0.95 μg/g). These facts suggest that the folate-deficient diet used in these studies (49,50) was in fact a diet containing supraphysiological supplemental levels of folate and that the control diet contained even higher levels of supplemental folate. This is supported by the comparison of plasma concentrations of homocysteine, an accurate inverse indicator of folate status, between these studies. The plasma homocysteine level in the folate-deficient diet group in these studies (49,50) was significantly lower than the usual level associated with the 0 mg of folic acid/kg diet as observed in the previous study (44) (4.2 vs. 7.26 μmol/L). This level of plasma homocysteine is in fact even lower than those levels achieved by supraphysiological folate supplementation (e.g., 4.34 μmol/L achieved by a diet containing 40 mg of folic acid/kg diet) (44). The plasma homocysteine concentration in the control group (8 mg of folic acid/kg diet) in these studies (49,50) was extremely low (1.7 μmol/L), suggesting that this diet contained extremely high levels of folate. Therefore, a more appropriate or correct conclusion from these studies (49,50) is that supraphysiological levels of folate supplementation enhance colorectal carcinogenesis, which is quite consistent with previous observations (44,46–48) as discussed before.

In summary, it appears that folate modulates colorectal carcinogenesis in chemical carcinogen rodent models over a wide range of dietary intakes. Folate deficiency of a moderate degree enhances colorectal carcinogenesis whereas modest levels of folate supplementation above the basal dietary requirement suppress colorectal tumorigenesis. Supraphysiological levels of folate supplementation do not appear to confer additional protection and, in some cases, may enhance colorectal carcinogenesis. The implication of this issue is important because the optimal dose of folate supplementation must be determined for folate chemoprevention to be effective and safe in humans. Although some similarities do exist, tumor development in chemical rodent models of colorectal cancer differs in several important histological, clinical, and molecular genetic aspects from that observed in humans, (53,54). Therefore, any extrapolation of the observations from these models to human situations should be made very cautiously.

Genetically predisposed murine models of intestinal tumorigenesis. Recently developed genetic murine models characterized by the spontaneous development of tumors of the small intestine and colon (54) have provided an excellent opportunity to investigate the role of folate in colorectal

carcinogenesis. One such model is Min (multiple intestinal neoplasia) mice, which carry a heterozygous germ-line mutation at codon 850 of the mouse *Apc* tumor suppressor gene and develop ~25–75 small intestine adenomas and 1–5 colorectal adenomas by 160–180 d, at which time they become moribund and die from anemia and intestinal obstruction (55,56). The number of small intestine adenomas usually reaches maximum in Min mice by age 3 mo. Phenotypic and genotypic features of this model resemble the syndrome of familial adenomatous polyposis coli in humans and hence Min mice have been extensively used to test the effects of potential chemopreventive agents and dietary factors.

A recently published study examined the effect of dietary folate deficiency and supplementation on the development and progression of small intestinal adenomas in Min mice (57). Min mice were randomly assigned to receive an amino acid–defined diet containing 0 (mild deficiency), 2 (control; basal daily requirement), 8, or 20 mg of folic acid/kg diet from weaning at ~21 d of age, and the number of small intestine adenomas was determined at 3 mo (i.e., at the time of maximum tumor development) and 6 mo (i.e., the effect of folate intervention on progression of established tumors). The results from this study indicate that dietary folate and serum levels of folate are inversely related to the number of small intestine adenomas at 3 mo of dietary folate intervention with the most consistent effect in the distal small intestine adenomas. The most profound protective effect on small intestine tumorigenesis was observed with the diet containing 10 times supplemental folic acid levels (i.e., 20 mg of folic acid/kg diet) above the basal dietary requirement, which reduced the number of adenomas by 68–78% compared with the other three folate groups. These observations corroborate findings from epidemiologic studies and animal experiments using chemical colorectal carcinogens in susceptible rats.

In contrast to the observations made at 3 mo, at 6 mo the mice fed 0 mg of folic acid/kg diet had 62–76% fewer distal small intestinal adenomas compared with the control and two folate-supplemented groups, although there were no significant differences in the numbers of total, proximal, and mid small intestine adenomas (57). Furthermore, serum folate concentrations were directly correlated with the number of distal small intestinal adenomas. This observation suggests that folate depletion might have caused regression of established distal small intestine adenomas in Min mice when dietary folate intervention was provided for an additional 3 mo (i.e., a total of 6 mo intervention) after adenomas were maximally established in the small intestine at age 3 mo. This inhibitory effect of folate depletion on established distal small intestine adenomas observed at 6 mo is consistent with the biochemical function of folate. Folate plays an important role in DNA synthesis and replication (58). Consequently, folate deficiency in tissues with rapidly replicating cells results in ineffective DNA synthesis. In neoplastic cells where DNA replication and cell division are occurring at an accelerated rate, interruption of folate metabolism causes ineffective DNA synthesis, resulting in inhibition of tumor growth (59). This has been the basis for antitumor therapy using a number of antifolate agents, including methotrexate, and 5-fluorouracil (59). Folate deficiency has indeed been shown to induce regression and suppress progression of preexisting neoplasms in experimental models (60–62). The addition of folate to established tumors has been shown to cause an acceleration phenomenon. For example, children with acute leukemia treated with folate supplementation experienced an accelerated progression of leukemia (63). Taken together, these observations suggest that folate deficiency has an inhibitory effect on progression of established neoplasms or may even cause regression of established tumors.

Although the Min mouse appears to be an excellent model for studying chemopreventive effects of dietary factors and drugs on intestinal tumorigenesis because of the spontaneous development of small intestinal polyps, genetic similarities to human colorectal cancer, and the accelerated nature of tumorigenesis, important limitations are associated with this model. First, the predominant phenotype in this model is the development of small intestine polyps in contrast to colon polyps in humans. Second, Min mice do not develop small intestine or colonic adenocarcinomas because they become moribund as a consequence of florid polyposis. Third, the contribution of aberrant crypt foci to the adenoma population in this model is not clearly established. Fourth, this model may reflect only inherited types of accelerated tumorigenesis, such as familial adenomatous polyposis, and not sporadic colorectal carcinogenesis. Last, the distal small intestine is the most susceptible whereas the colon is relatively resistant to chemopreventive actions of drugs and nutritional factors in this model. Because of this site-specific susceptibility of chemoprevention, the extrapolation of information concerning the effects of chemopreventive agents in this model to humans is therefore uncertain. Notwithstanding the limitations associated with this model, the data from Min mice suggest that dietary folate supplementation suppresses the development of small intestine adenomas in a dose-responsive manner in this murine model but folate status might have an opposite effect on established small intestine adenomas. A recent study that used a casein-based diet deficient in folic acid and choline did not show a consistent effect of folate deficiency on small intestine tumorigenesis in Min mice (64).

The observations from Min mice that suggest that the timing of folate intervention may be important in providing safe and effective folate chemoprevention have led to another animal study using *Apc*+/−*Msh2*−/− mice (65). *Apc*+/−*Msh2*−/− were generated by crossing Min mice with *Msh2*−/− mice (66). The *MSH2* gene is one of several mismatch repair genes that ensure accurate replication of the genome during cell division (36). Germ-line mutations in the mismatch repair genes have been implicated in hereditary nonpolyposis colorectal cancer syndrome and somatic mutations in ~15% of sporadic colorectal cancers (36). Mutations in the mismatch repair genes result in a mutator phenotype in which loss of postreplicative DNA repair increases the mutation rate and results in a replication error phenotype or microsatellite instability (36). *Apc*+/−*Msh2*−/− mice display an accelerated intestinal adenoma phenotype and develop numerous dysplastic colonic aberrant crypt foci (66). They develop ~350 small intestine adenomas, eight colonic adenomas, and 55 aberrant crypt foci by age 80 d, at which time they become moribund and die from anemia or bowel obstruction. The average time required for a nascent tumor to develop into a macroscopically visible adenoma in these mice was estimated to be 42 d in the small intestine. Because of the accelerated nature of tumorigenesis in this model, it is possible to test the effects of folate on initiation and progression of small intestinal polyps in a relatively short time.

Apc+/−*Msh2*−/− mice were randomly assigned to receive an amino acid–defined diet containing either 0 (mild deficiency) or 8 mg (sufficiency) of folic acid/kg diet starting at either 3 or 6 wk of age and the tumor development was assessed at 11 wk of age (65). Given that the average time required for a nascent tumor to develop into a macroscopically visible adenoma in these mice was estimated to be 42 d in the small intestine (66), 3 wk was chosen to represent a time before the

establishment of neoplastic foci; 6 wk was chosen to represent the time after the establishment of neoplastic foci. This study clearly showed that the dietary folate has strikingly different effects on intestinal tumorigenesis in *Apc*+/−*Msh2*−/− mice depending on the timing of intervention. Folate supplementation at four times the basal dietary requirement for rodents, started before the establishment of neoplastic foci, significantly decreased the number of small intestine adenomas threefold over the diet with a moderate degree of folate deficiency (65). In contrast, dietary folate had an opposite effect on the development of small intestine adenomas when given after the establishment of neoplastic foci. In this situation, moderate folate deficiency induced by dietary depletion of folate significantly decreased the number of small intestine adenomas by 4.2-fold compared with folate supplementation at four times the basal dietary requirement (65). In addition, dietary folate supplementation, started before the establishment of neoplastic foci, significantly decreased the number of colonic aberrant crypt foci and adenomas by 2.8-fold and 67%, respectively, compared with a moderate degree of folate deficiency, whereas no significant effect on colonic aberrant crypt foci and adenomas was observed with dietary folate intervention started after the establishment of neoplastic foci (65). Therefore, these observations suggest that the timing of folate intervention is critical in providing an effective and safe chemopreventive effect on colorectal carcinogenesis and that folate intervention, at modest supplemental levels, should be started before the establishment of neoplastic foci to prevent colorectal cancer. The *Apc*+/−*Msh2*−/− murine model is associated with limitations: the predominant phenotype in this model is the development of small intestine adenomas in contrast to colon polyps in humans; *Apc*+/−*Msh2*−/− mice do not develop adenocarcinoma of the small intestine or colon because they become moribund as a consequence of florid polyposis; the contribution of aberrant crypt foci to the adenoma population in this model is not clearly established; this model may reflect only inherited types of accelerated tumorigenesis, such as familial adenomatous polyposis and hereditary nonpolyposis colorectal cancer syndrome, and not sporadic colorectal carcinogenesis; and, despite Msh2 deficiency, widespread microsatellite instability is absent in tumors. Notwithstanding these limitations, the data from *Apc*+/−*Msh2*−/− mice suggest that the optimal timing of folate intervention should be established before folate supplementation can be used as a safe chemopreventive agent against colorectal cancer.

The effect of dietary folate on breast cancer development in an animal model. Two recently published studies that investigated the effect of dietary folate on the development of mammary tumors in the well-established *N*-methyl-*N*-nitrosourea (MNU) rat model of breast cancer also confirmed the importance of timing of folate intervention in carcinogenesis (67,68). In these studies, female Sprague-Dawley rats were randomly assigned to receive an amino acid–defined diet containing either 0 (mild deficiency), 2 (control; basal dietary requirement), or 8 mg of folic acid/kg diet and MNU was used to induce mammary tumorigenesis. Dietary folate intervention was manipulated to determine the effect of folate on the initiation and promotion phases of MNU-induced mammary tumorigenesis. These studies collectively demonstrated that moderate dietary folate deficiency significantly suppresses whereas folate supplementation at four times the basal dietary requirement does not significantly modulate mammary tumorigenesis in this model. No effect of dietary folate was observed in the initiation phase whereas the inhibitory effect of dietary folate deficiency was in the promotion and progression phase of MNU-induced mammary tumorigenesis. This observation suggests that the conventional dose and route of MNU injection used in these studies created an overwhelmingly carcinogenic milieu for folate status to modulate initiation of mammary tumorigenesis. Regardless of the levels of dietary folate, MNU induced and established neoplastic foci in mammary tissues. In this setting, folate deficiency suppressed the progression of or caused regression of established mammary neoplastic foci. This explanation is consistent with the prior observations made in Min and *Apc*+/−*Msh2*−/− mice (57,65). Therefore, the inhibitory effect of folate deficiency on MNU-induced mammary tumorigenesis in this rat model might have been primarily on promotion and progression of established mammary neoplastic foci.

Animal model of colorectal dysplasia and cancer associated with ulcerative colitis. The potential effect of folate supplementation on colorectal carcinogenesis in chronic ulcerative colitis was investigated in a recently developed and characterized genetically predisposed murine model, the interleukin-2 and β_2-microglobulin deficient (IL-2^{null} × β_2m^{null}) mouse (69). IL-2^{null} × β_2m^{null} mice develop mild-to-moderate colitis with diarrhea, mild wasting, and some rectal prolapse, usually between 8 and 12 wk; most mice recover with normal stool, weight gain, and normal appearance and survive beyond 6 mo, suggesting active disease followed by remission from colitis (70). Histologically, 75% of these mice have mild-to-moderate colonic inflammation of the entire colon and 25% have no inflammation at the time of necropsy (70). Some of the IL-2^{null} × β_2m^{null} mice develop well- to moderately differentiated adenocarcinoma in the proximal half of the colon between 6 and 12 mo (71). The molecular genetics of colorectal cancer arising in this model are sufficiently similar to those of human sporadic and ulcerative colitis–associated colorectal cancer (72). Weaning IL-2^{null} × β_2m^{null} mice were randomly assigned to receive 0 (mild deficiency), 2 (basal dietary requirement, control), or 8 mg (supplemented) of folic acid/kg diet for 32 wk (69). 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% vs. 65.1%, $P = 0.009$). Interestingly, the incidence of high-grade lesions in the folate-deficient group was also 49% lower than that in the control group (33.3% vs. 65.1%, $P = 0.007$). The higher mortality rate in the folate-deficient group than in the other two groups (25% vs. 6.5% and 5.6%, respectively, $P < 0.02$) partially accounted for the low incidence of high-grade lesions in this group. These data indicate that dietary folate supplementation at four times the basal dietary requirement significantly suppresses colorectal carcinogenesis associated with ulcerative colitis in this model. These data also suggest that folate deficiency may inhibit colorectal carcinogenesis in chronic ulcerative colitis, although the high mortality observed in the folate-deficient group precludes a definitive conclusion.

What have we learned from animal models concerning the effect of folate status on colorectal carcinogenesis? Although animal studies generally support a causal relationship between folate depletion and colorectal cancer risk, these studies show that the dose and timing of folate intervention are critical in providing safe and effective chemoprevention; exceptionally high supplemental folate levels and folate intervention, after microscopic neoplastic foci are established in the colorectal mucosa, promote rather than suppress colorectal carcinogenesis. These animal studies in conjunction with clinical observations suggest that folate possesses the dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention. Folate deficiency has an inhibitory effect whereas folate supplementation has a promoting effect on the pro-

gression of established neoplasms. In contrast, folate deficiency in normal epithelial tissues appears to predispose them to neoplastic transformation, and modest levels (4–10 times above the basal dietary requirement) suppress whereas supraphysiological doses enhance the development of tumors in normal tissues.

Important issues concerning folate chemoprevention

Although folate appears to be an ideal candidate for chemoprevention given its proven safety and cost (73), the safe and effective dose range of folate supplementation and optimal timing of folate chemoprevention have not been clearly established in humans. Animal and some clinical studies have suggested that folate supplementation may increase cancer risk and accelerate tumor progression if too much is given or if it is provided after neoplastic foci are present in the target organ. Therefore, it appears that modest levels of folate supplementation should be implemented before the development of precursor lesions in the target organ or in individuals free of any evidence of neoplastic foci. It is, however, obvious that determining the presence of neoplastic foci in the general population is an almost impossible task. Furthermore, what constitutes safe precursor or preneoplastic lesions for which folate may exert a protective effect while preventing their progression to frank neoplastic lesions has not yet been established. For example, should folate chemoprevention be started before there is evidence of established aberrant crypt foci or microscopic adenomatous neoplastic lesions in the colorectum or can it be started even after these lesions are present? Recent advances in molecular biology may be able to clarify this issue by identifying certain molecular and genetic markers within these lesions that may predict their potential to progress to more advanced neoplastic lesions.

Folate chemoprevention has a very important public health implication. As discussed briefly above, evidence for a protective effect of folate supplementation on neural tube defects (4,5) was considered to be sufficiently conclusive and led to mandatory folic acid fortification in the United States (9) and Canada (10) in 1998. The effectiveness of folic acid fortification in improving folate status has already been shown to be quite striking with a dramatic increase in blood measurements of folate (serum, plasma, and red blood cell) and a substantial decrease in plasma homocysteine levels in the United States and Canada (74–79). Preliminary reports suggest a significant reduction in the prevalence and incidence of neural tube defects in the United States and Canada (80–82). However, the long-term effect of folic acid fortification on the risk of colorectal and other cancers may not be as clear as the one observed in regards to neural tube defects. Although folic acid fortification may prevent the development of cancers in individuals without preexisting premalignant lesions or neoplastic foci, it may promote the progression of these lesions in individuals harboring them. With respect to colorectal cancer, adenomas are found in ~25% of people by age 50 y in the United States and the prevalence increases with age (42). An estimate based on autopsy series, which are probably less susceptible to selection and detection bias than clinical series, is that the prevalence of adenomas is higher in men than women at any given age, reaching ~60% in men and ~40% in women by the age of 50 y (42). Given the prevalence of colorectal adenomas in the general population in the United States, whether or not folate fortification promote the progression of adenomas to adenocarcinomas in the colorectum is a legitimate public health concern and needs careful monitoring.

A more logical approach to folate chemoprevention might be that of targeted chemoprevention in individuals at high risk of developing colorectal cancer without evidence of preexisting premalignant lesions or neoplastic foci. For instance, individuals with the MTHFR 677 TT genotype with inadequate folate intake or with significant alcohol consumption have been shown to have an increased risk of colorectal cancer (17,18,20,21). These individuals may therefore benefit from targeted folate chemoprevention once the colorectum is considered to be free of premalignant lesions or neoplastic foci.

Mechanistic understanding of the folate deficiency-mediated colorectal carcinogenesis

Epidemiologic and experimental evidence indicating a causal association between folate and colorectal cancer is strengthened when biological pathways or mechanisms by which colorectal carcinogenesis may be modified are identified and when these mechanisms are biologically plausible (52). It can be argued that epidemiologic data, however strong and consistent, are an inadequate basis for any definite judgment of causality unless supported by mechanistic evidence (52). For folate, potential mechanisms by which deficiency enhances and supplementation suppresses colorectal carcinogenesis exist and have been extensively reviewed (**Table 1**) (11,12,83–85). The mechanisms by which dietary folate can modulate colorectal carcinogenesis are related to the sole biochemical function known for folate: mediating the transfer of 1-carbon moieties (Fig. 1) (58). In this role, folate is an important factor in DNA synthesis, stability and integrity, and repair (Fig. 1), aberrations of which have been implicated in colorectal carcinogenesis (11,12,83–85). Folate may also modulate DNA methylation (Fig. 1), which is an important epigenetic determinant in gene expression (an inverse relationship), in the maintenance of DNA integrity and stability, in chromosomal modifications, and in the development of mutations (11,12,83–85). 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 (11,12,83–85). Although genomic and site-specific DNA hypomethylation has been considered as a leading mechanism by which folate depletion enhances colorectal carcinogenesis (11,12,83–85), this hypothesis remains highly controversial and unresolved (86–94). A mechanistic understanding of how folate status modulates colorectal carcinogenesis further strengthens the case for a causal relationship and provides insight into a possible chemopreventive role of folate.

The role of folate has greatly evolved over the past two decades from the prevention of anemia to the prevention of cardiovascular disease and neural tube defects. Accumulating

TABLE 1

Potential mechanisms of the folate deficiency-mediated colorectal carcinogenesis

- Aberrant genomic and site-specific DNA methylation
- DNA damage, uracil misincorporation, impaired DNA repair
- Increased mutagenesis
- Hyperproliferation
- Abnormal apoptosis
- MTHFR polymorphisms and related gene-nutrient interactions

evidence suggests that folate may also play a role in the prevention of cancer. This expanding role of folate nutrition in cancer prevention has major public health implications, and future research needs to clearly elucidate the effect of folate on carcinogenesis. Although the jury is still out, the implications for folate chemoprevention of cancer remain provocative.

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