The Mechanisms by Which Folate Depletion Enhances Colorectal Carcinogenesis: A Unified Scheme

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Recent reviews in the epidemiologic literature have demonstrated the remarkably consistent association between the low consumption of fruit and vegetables and the incidence of cancer [1, 2]. Many components of fruits and vegetables may be responsible for the reduced risk of cancer, such as vitamins, other micronutrients, plant phenolics, fiber, as well as the low saturated fat and calorie content of such foodstuffs. Among these candidate components is folate, a depletion of which is increased among people who eat few fruits and vegetables. Folate is an attractive candidate in this regard because of its important roles in DNA metabolism. The similar morphologic features of folate-deficient (“megalocytic”) and premalignant (“dysplastic”) cells further underscores this relationship.

Epidemiologic studies have suggested that diminished folate status is associated with cancer of the cervix, colorectum, lung, esophagus, brain, pancreas, and breast. Among these, epidemiologic support for such a relationship is most compelling for colorectal cancer [reviewed in 3]. Folate deficiency also has been considered as an important factor in alcohol-related carcinogenesis because alcohol alters normal folate metabolism in a number of ways [4].

Preeminent among the limitations of the epidemiologic literature, however, is its inability to prove causality. In addition, nearly all the epidemiologic studies to date have used dietary and/or blood folate levels as the index of folate status; such measures do not necessarily reflect folate concentrations in the tissue of interest. This is potentially of considerable importance since recent studies suggest that the
susceptibility to folate depletion varies widely between tissues [5]. Aberrancies in the distribution of the different coenzymatic forms of folate also might be as important a factor as total tissue folate concentration and have, to date, not been evaluated.

Controlled experiments in animal models of cancer have generally complemented the epidemiologic studies, although the results are not entirely consistent. Cravo et al. [6] demonstrated that rats treated with dimethylhydrazine are more likely to develop microscopic foci of colonic dysplasia and adenocarcinoma when fed a diet low in folate than when fed a replete diet. Kim et al. [7] similarly demonstrated the protective effect of folate supplementation against the development of macroscopic colonic neoplasms. In contrast, Baggott et al. [8], Wargovich et al. [9], and Poirier [10] did not find any protective effects of folate supplementation on carcinogenesis in mammary, colon and hepatic tumors, respectively. The fact that the existing animal studies are conflicting is not surprising when one recalls that carcinogenesis in animals depends on several factors, including the species, the tumor model and type, the timing, dose, and length of application of carcinogen, the stage of carcinogenesis, the dietary level and form of folate administered, as well as its chronologic relationship to carcinogen administration.

Human intervention trials, designed to determine whether individuals at increased risk of cancer have that risk reduced by supraphysiologic doses of folate, have been performed almost exclusively in regard to cancer of the uterine cervix and colorectum [reviewed in 11]. All such studies to date have used intermediary biomarkers rather than cancer incidence itself as primary endpoints. In the case of the cervix, the grade of dysplasia is usually the intermediary marker used, whereas in the case of the colorectum, histologic, molecular and cell proliferation indices have each been utilized as intermediary biomarkers of cancer. Butterworth et al. [12] reported that folate supplementation was associated with a significant degree of regression in cytologic and histologic evidence of dysplasia in a randomized group of 47 women. However, in a more recent study by the same investigators, which enrolled a larger number of subjects, the positive results were not reproduced [13]. Several small, randomized intervention trials have been published in the field of folate and colon cancer; the results have been rather promising but the rather modest size of the populations studied as well as other limitations of the studies preclude any definite statement about the potential utility of folate as a cancer chemopreventive agent [reviewed in 11]. Four large, randomized multicenter trials are underway in the US and will likely provide more definitive answers to this question in the next several years.

The remainder of this review will focus on the various mechanisms that have been described which appear to constitute the means by which folate depletion enhances carcinogenesis. The various mechanisms described are intimately interrelated and therefore an attempt has been made to develop a unified scheme that explains the complex web of effects which mediate the procarcinogenic potential of folate depletion.
Folate in Nucleic Acid Metabolism

The sole biochemical function of all of the coenzymatic forms of folate in mammalian systems appears to be mediating the transfer of one-carbon units. Within the scope of this function is the synthesis of S-adenosylmethionine, a methyl donor used widely for biological methylation reactions, and de novo deoxynucleoside triphosphate synthesis: each of these two biosynthetic pathways are a means by which folate plays a major role in DNA metabolism. It is through disturbances in normal DNA, and possibly RNA, metabolism that folate depletion appears to produce its procarcinogenic effects.

Methionine is regenerated from homocysteine in a reaction catalyzed by 5-methyltetrahydrofolate (5-methylTHF):homocysteine methyltransferase: this is a reaction for which 5-methylTHF serves as both a cofactor and substrate (Fig. 1). An alternative mechanism for the regeneration of methionine which does not require folate also exist – the methylation of homocysteine by betaine – although the latter reaction seems only to be operative in the liver and kidney. Methionine, in turn, is converted to S-adenosylmethionine (SAdoMet) in a reaction catalyzed by methionine adenosyl transferase. SAdoMet then donates the labile methyl group it derived from 5-methylTHF for over 80 biological methylation reactions, including an array of reactions whereby specific sites within DNA and RNA become methylated. Although the alternative betaine pathway may partially compensate [14], it is nevertheless well known that dietary folate depletion alone is a sufficiently perturbing force to diminish SAdoMet pools [15].
The synthesis and turnover of deoxynucleoside triphosphate (dNTP) pools are tightly coupled to DNA synthesis. Since dNTPs are the immediate substrates for the polymerases involved in DNA replication and repair, the fidelity of DNA synthesis is critically dependent on the correct balance and availability of deoxynucleotides. Folate-derived one-carbon groups are essential for the de novo synthesis of the pyrimidine, thymidylate, as well as the purines. In mammalian cells the de novo synthesis of thymidylate (dTMP) from deoxyuridylate (dUMP) is a rate-limiting step for DNA synthesis and requires 5,10-methylenetetrahydrofolate as a coenzyme.

When the dietary methyl supply is inadequate, such as in folate depletion, the use of folate coenzymes for biological methylation and nucleotide synthesis appears to compete. As SAdoMet concentrations decrease, compensatory mechanisms increase the conversion of 5,10-methyleneTHF to 5-methylTHF, an irreversible reaction, and thereby compromise folate availability for de novo nucleotide synthesis [16].

Candidate Mechanisms for Folate-Associated Carcinogenesis

Altered DNA Methylation

In vertebrate genomes, approximately 4% of cytosine residues are modified post-synthetically to 5-methylcytosine (5mC). Most of these 5mC residues are found in the palindromic sequence, CpG. The cell strictly maintains its particular patterns of methylated residues, although transient changes in methylation occur within promoter sites for certain genes in conjunction with altered expression of the genes. Similarly, the pattern is precisely inherited when mitosis occurs [17]. Such meticulous maintenance underscores the important roles that DNA methylation is thought to play in the regulation of gene expression and gene integrity.

There is considerable evidence that aberrant DNA methylation plays an integral role in oncogenesis. First, a decreased level of genomic methylation is a nearly universal finding in tumorigenesis: this has been observed in cancers of the colon, stomach, uterine cervix, prostate, thyroid and breast [reviewed in 11]. This decrease in genomic methylation appears early in carcinogenesis, and appears to precede more well-described mutation and deletion events that occur later in the evolution of cancer. Genomic hypomethylation has been observed in some animal models of carcinogenesis as well [18]. Gene-specific hypomethylation may occur even in the absence of genomic hypomethylation and is probably a more important event in carcinogenesis since the prevailing theories of carcinogenesis emphasize damage which occurs at critical sites within DNA. Site-specific aberrancies in DNA methylation within critical genes are also observed in neoplastic tissues, and include both foci of hypomethylation and hypermethylation [17].

Somewhat surprisingly, the patterns of DNA methylation that are so religiously guarded by the cell seem to be susceptible in certain settings to perturbations
created merely by altering dietary folate: this has been observed in both animals and man. Jacob et al. [19] demonstrated the induction of genomic hypomethylation in human lymphocytic DNA when healthy human volunteers were placed on a long-term folate-deficient diet and showed that the effect was reversible when the deficiency was corrected. Supportive evidence from a recent observational study exists as well: Fowler et al. [20] reported that the serum folate level as well as folate concentrations in the uterine cervix were significantly correlated with genomic DNA methylation in a study of cervical intraepithelial neoplasia. Studies performed in rodents fed diets deficient in folate generally do not show any changes in genomic DNA methylation, although it does appear to be feasible with a severe deficiency state or one deficient in multiple lipotropes such as choline, methionine, vitamin B12, and folate [18]. The resistance to the induction of genomic methylation in rats may be due to the fact that they have a more active betaine pathway than humans.

As mentioned above, the induction of site-specific hypomethylation may be more critical to the process of carcinogenesis than genomic effects. In this regard, folate depletion has been shown to induce hypomethylation of the coding region of the p53 tumor suppressor gene even in the absence of genomic hypomethylation [21]. Conversely, supplemental folate has been shown to revert the hypomethylation of this region which occurs in association with chemical carcinogenesis [22]. Of particular interest is the fact that this region within the p53 gene that is particularly susceptible to hypomethylation by folate depletion or chemical carcinogens (exons 5–8) is precisely that region most frequently mutated in human cancer [23].

Recent studies reveal why changes in site-specific methylation may be related to subsequent mutations at that site. 5mC is more unstable than its unmethylated counterpart. Hydrolytic deamination of 5mC leads to a G/T mismatch and subsequently, if unrepaired, to a C→T transition mutation [24, 25]. This probably explains why sites of DNA methylation are mutational hotspots in many human tumors. Paradoxically, unmethylated cytosine can also undergo deamination to yield uracil, particularly under conditions where intracellular SAdoMet is low (such as in folate deficiency) [26]. The repair enzyme, uracil-DNA glycosylase efficiently repairs G:U, but not G:T mismatches. However, DNA-methyltransferase may block this repair or other conditions may impair the repair process and thereby lead to C→U→T transition mutations [27]. In our studies [21, 22] in rodents, we have found that dietary folate depletion produces diminished methylation in the so-called “hypermutable region” of the p53 gene (exons 5–8); a region where 24% of reported mutations occur at C→T transitions at CpG dinucleotides. This suggests that the phenomenon of DNA methylation may contribute to these mutations [28].

Transcriptional repression by hypermethylation of promoter sequences has been widely discussed as an alternative means for the inactivation of tumor-suppressor genes in cancer: methylation-induced alterations in the local confor-
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mation of the gene can render it inaccessible and transcriptionally inactive and is the presumptive mechanism involved. Hypermethylation of the promoters of p16, calcitonin, and estrogen receptor genes have all been observed in neoplastic tissue [29, 30]. Early reports suggested this was due to increased DNA-methyltransferase activity in cancers, although recent reports suggest that methyltransferase activity is not truly elevated in neoplastic tissue when the data are corrected for the proliferation rate of the tissue [31]. Pogribny et al. [32] demonstrated that a lipotrope-deficient diet can paradoxically induce hypermethylation at selected sites in the genome: they observed progressive exon-specific hypomethylation of the hepatic p53 gene in animals on a diet deficient in folate, B12, methionine and choline, followed by a rebound hypermethylation at a later time when neoplastic foci became histologically evident in the liver. Direct evidence that isolated folate deficiency can similarly produce hypermethylation of critical tumor-suppressor gene promoter regions is lacking to date and needs to be examined.

Altered RNA Methylation

Like DNA, a wide variety of RNA species are methylated at specific sites by SAdoMet-mediated reactions. In some instances, the 5'-methyl cap of RNA is methylated and in other instances, internal nucleotide residues are methylated. Although the precise functions of RNA methylation sites are only now becoming apparent, it appears that these patterns of methylation in RNA are also judiciously guarded by the cell and serve important functions in maintaining the stability of the RNA species and facilitating transport across the nuclear membrane [33]. Demethylation of tRNA was shown some years ago with a severe, methyl-deficient diet [34]. Only recently, however, has it been shown that folate depletion alone (at least in cell culture) is sufficient to demethylate some RNA species such as small nuclear RNA [35], a species which is a critical component of the machinery necessary for maturation of messenger RNA. Whether this alters maturation of mRNA for critical tumor suppressor genes is presently under investigation.

Disruption of DNA Integrity

It has been known for some years that folate deficiency induces breaks in chromosomes and that such breaks are associated with an increased risk of cancer in humans. More recently, folate-deficient conditions in both cell culture and animal experiments have been shown to create an excess of breaks in the phosphodiester backbone of DNA, which is presumed to be the molecular basis for chromosomal breaks [36, 37]. There are several mechanisms by which folate deficiency might create such breaks: these include the incorporation of uracil from the cellular nucleotide pool into DNA and by in situ deamination of cytosine [38]. Folate deficiency reduces dTMP synthesis from dUMP and the ensuing nucleotide imbalance increases the misincorporation of uracil bases into DNA; this is due to the fact that most DNA polymerases do not effectively distinguish between dUMP and dTMP [39]. Uracil in DNA is excised by a repair glycosylase, and in
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the process a transient single-strand break develops in the DNA. Simultaneous removal and repair of two adjacent uracil residues on opposite strands can result in a double-strand DNA break, further exacerbating genetic instability. Unrepaired double-strand DNA breaks enhance cellular transformation in culture and increase cancer risk. Excessive DNA uracil content, as well as increased numbers of chromosomal breaks, are observed in folate-deficient humans, and both defects are reversed by folate administration [40]. Fenech et al. [41] also found that folate supplementation above the recommended daily allowance was observed to lessen chromosome breakage below levels observed in normal, folate-replete individuals.

Studies in systems other than the human subject also support such an effect of folate depletion. In a cell culture study, folate-deficient media enhanced DNA strand breaks induced by an alkylating agent and γ-irradiation [36] and, in a recent rodent study, a folate-deficient diet increased gene-specific DNA strand breaks in the hypermutable region of p53 [21]. Site-specific hypomethylation was also noted at this site, thereby supporting the speculation that deamination of non-methylated C turns to uracil and removal of uracil induces strand breaks.

The induction of strand breaks has been observed to create chromosomal aberrations evident at mitosis, an increase in mutation rates, as well as in the development of neoplastic transformation [reviewed in 11]. However, the functional significance of DNA strand breaks induced by folate deficiency is yet not known with certainty.

In those instances where cancers are enhanced by particular viruses, the phenomena of hypomethylation and strand breaks may have an additional significance. For instance, human papilloma virus 18 is widely accepted as a risk factor for human cervical neoplasia. It is incorporated into the human genome at four loci, three of which are in or near a constitutive “fragile site” that is created by folate depletion. More recently, integrated human polyomavirus JCV DNA sequences have been identified in human colon cancer DNA, raising the question as to whether the virus plays an etiologic role [42]. Methylation of specific sites is known to block the integration of certain viruses into the genome and strand breaks are thought to perhaps enhance integration. Whether the hypomethylation or strand break sites produced by folate deficiency might enhance the incorporation of tumorigenic viruses remains a provocative concept.

Disruption of DNA Repair

Although DNA is the carrier of genetic information, it has limited biochemical stability. It is constantly damaged by a host of endogenous and exogenous factors, and therefore sophisticated repair mechanisms are available in all cells to eliminate such damage.

As mentioned above, folate deficiency induces dNTP pool imbalance and uracil misincorporation into DNA. Such misincorporation results in abnormal DNA replication and imposes greater dependence on the repair system. In an in vitro study, Chinese hamster ovary (CHO) cells grown in folate-deficient media
showed various types of chromosomal aberrations, but cells grown in hypoxanthine-supplemented folate-deficient medium exhibited a significantly lower frequency of damaged mitotic figures [43]. Hypoxanthine is a purine precursor which bypasses the need for folate-dependent purine biosynthesis. In another CHO cell study, folate deficiency acted synergistically with alkylating agents to increase somatic mutations and, with \( \gamma \)-irradiation, to promote DNA strand breaks, by limiting DNA repair [36]. In a folate-deficient rodent model we found that the DNA excision repair was impaired in folate-deficient colonic mucosal cells compared to normal mucosal cells [44]. In this study supplementation of the colonocytes with hypoxanthine as a purine precursor and thymidine as a pyrimidine precursor, which together preclude the need for folate-dependent nucleotide synthesis, partially reversed the impaired excision repair. This suggests that folate deficiency disrupts excision repair in part by altering the cellular pool of deoxyribonucleotides. Similarly, Duthie and Hawdon [45] found diminished DNA repair capability of human lymphocytes in a folate-deficient medium. Since the p53 gene product is an important regulator of DNA repair and the cell cycle, impairment of DNA repair in the setting of folate depletion might feasibly be mediated in part by the adverse effects that depletion has on the integrity of the p53 gene [21].

Recently, Cravo et al. [46] reported that folate deficiency might impair the other major cellular DNA repair system, mismatch repair, in ulcerative colitis patients. They suggested that increased microsatellite instability in these patients might translate into an increased risk for mutations. Also, the methylation of CpG sites in the hMLH1 gene, one of the major mismatch repair genes, has been associated with microsatellite instability in colon cancer and stomach cancer [47].

The state of DNA methylation, which can be altered by folate depletion as mentioned above, plays an important role in strand discrimination during post-replication mismatch repair [48]. Methylation of DNA by sequence-specific methylases lags behind the replication fork. Thus, immediately after synthesis, the newly synthesized daughter strand is undermethylated relative to the parental strand. This difference in methylation state between the parental and daughter strands behind the replication fork permits discrimination between the two strands. With hemimethylated heteroduplexes, which are methylated at GATC sequences only on one DNA strand, repair is highly biased to the unmethylated strand, with the methylated strand serving as the template for correction [49]. Therefore, site-selective DNA hypomethylation induced by folate deficiency might affect methyl-directed mismatch repair.

**Methylenetetrahydrofolate Reductase and Incidence of Colon Cancer**

Methylenetetrahydrofolate reductase (MTHFR) catalyzes the irreversible conversion of 5,10-methyleneTHF to 5-methylTHF. A common polymorphism of this gene (C677T) causes thermolability and reduced activity of MTHFR. Over
the past few years, two studies have reported that men with the homozygous mutation have half the risk of colorectal cancer compared with the homozygous wild-type or heterozygous genotypes [50, 51]. Among men with adequate folate levels, a threefold decrease in risk was observed. However, protection associated with the mutation was largely absent in men with a low systemic folate status. It was suggested that the cancer-protective effect of the MTHFR mutation was related to increased availability of 5,10-methyleneTHF, and therefore increased the ease of nucleotide synthesis. One might speculate that, under low but not high folate conditions, the availability of 5-methylTHF for biological methylation constitutes a more critical determinant of whether the cell is pushed down the pathway towards neoplasia [51].

Recently Bagley and Selhub [52] found that human subjects possessing the homozygous polymorphism had formylated forms of THF in their red cells; this compares with wild-type individuals, whose cells contain only methylTHF. In a preliminary study, we have also found that lymphocytic DNA from subjects with the MTHFR polymorphism is significantly less methylated than DNA from wild-type subjects [53]. These observations suggest that the protective effect of the polymorphism may be conveyed by an alteration in the forms of folate available contained within the cell, and it explains how the protective effect might be operable even when total folate levels are normal.

Figure 2 summarizes the molecular effects of folate depletion that are described in this chapter and provides a framework of how these phenomena are interrelated. Although it is an oversimplification, increased DNA damage without a compensatory increase in DNA repair and aberrant expression of critical genes are generally agreed upon to be major pathways towards cancer. Figure 2
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outlines how all of these effects synergistically contribute to increased DNA damage and alterations in gene expression. At this point in time, the schema presented in Figure 2 has not been conclusively proven to constitute the means by which folate depletion enhances cancer; nevertheless, considerable work is presently underway that should give us a more definitive answer in the near future.

Conclusion

An expanding body of epidemiologic, animal and human studies suggest that folate status modulates the risk of developing cancers in several tissues: folate depletion appears to enhance carcinogenesis and folate supplementation conveys a protective effect. The mechanistic studies performed to date have not definitively defined the mechanism(s) that are responsible for mediating this effect, although we are rapidly approaching such an understanding. Since folate is a critical element for both DNA methylation and synthesis, and because aberrations in DNA are widely held to be the origin of most carcinogenic processes, most studies have focused on these effects. Interestingly, alterations in DNA methylation, disruption of DNA integrity caused by increased uracil misincorporation or DNA-strand breaks, and disruption of DNA repair are related phenomena that can each be induced by folate depletion and are believed to enhance carcinogenesis by altering the expression of critical genes. Most recently, the role of a common polymorphism of the MTHFR gene has been highlighted as well, because its presence is associated with a remarkable decrease in the incidence of colorectal cancer. This review presents a unified scheme as to how folate insufficiency might enhance carcinogenesis through these interrelated phenomena.

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Discussion

Dr. Tisdale: I didn’t really understand your logic on the mechanism of hypomethylation, because most of the methionine or S-adenosylmethionine comes either from the diet or through the liver, through betaine homocysteine methyltransferase. Therefore a lack of folate won’t necessarily lead to a methyl depletion. In fact, the homocystine methyltransferase is thought mainly to convert the 5-methyltetrahydrofolate in the diet into tetrahydrofolate which can be used for nucleotide biosynthesis, and therefore you get megaloblastic anemia in the case of vitamin B₁₂ deficiency. I wondered if it has anything to do with the carcinogen that you were using, which was dimethylhydrazine, because that would itself be metabolized to methyl groups, and folate could actually act as a sink and protect the DNA from methylation. So you’d see increased methylation from your carcinogen, which in itself would lead to hypermethylation of the DNA.

Dr. Mason: First of all, let me address the issue of folate and DNA hypermethylation. You’re right that, depending upon the particular situation, a lot of the methyl input into the ‘methyl cycle’ is from dietary methionine. But let me also remind you that there are several animal studies, and even human studies, that show that if you put an organism on a folate-depleted diet you can decrease levels of S-adenosylmethionine. So apparently there is not full compensation in that cycle from input from dietary methionine and from the other sources in that cycle to compensate for chronic folate depletion. It’s pretty clear that if you put animals on a folate-deficient diet, S-adenosylmethionine levels drop. In fact in our animal studies we’ve shown that both in the liver and in the colon folate depletion alone, even moderate folate deficiency, will cause significant falls in S-adenosylmethionine levels. Now in terms of the betaine enzyme, remember that this is only present in the liver and the kidney; it’s not present in the colon. Although you’re correct that the betaine pathway could feasibly bypass folate deficiency, nevertheless because it doesn’t exist to any significant degree in the colon it doesn’t seem to be able to compensate in that particular organ.

As to your observation about dimethylhydrazine, that’s a reasonable criticism of this animal model. The reason we decided to use it was because, as you’re probably aware, the dimethylhydrazine model is by far the most commonly used model of colonic carcinogenesis in the animal. We have done studies – which I haven’t shown here today – to try to address some of these doubts. For instance, there was concern when we initially started doing these studies that folate depletion might alter the activation of dimethylhydrazine in the colon; indeed the reason it is a colon-specific carcinogen is that the bacterial flora actually activates the carcinogen. We did studies looking at the active metabolites of dimethylhydrazine and showed that the imposition of a folate-deficient diet really didn’t alter the development of these active metabolites of dimethylhydrazine, which are the proximate carcinogens of the colon. Dimethylhydrazine operates by alkylating nucleotides; another thing that we’ve shown is that dimethylhydrazine itself causes DNA hypomethylation by alkylating nucleotides in DNA. What it does is to block the ability of DNA methyltransferases to methylate DNA. If you look at DNA methylation in animals that have been treated with dimethylhy-
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drazine, you see fairly significant DNA hypomethylation. You could even argue that the means by which dimethylhydrazine induces cancer is by inducing DNA hypomethylation. It does get rather complicated trying to dissect out all these different effects, so I agree with you that there are limitations of this animal model, as there are for all animal models.

Dr. Kho: Apart from hypomethylation, it is known that there are certain areas in genome cancer that are hypermethylated. What is the mechanism of this? Do you think folate is involved? Or DNA methyltransferase?

Dr. Mason: That’s a very interesting question. My laboratory and several others are looking at DNA hypomethylation, but there are probably more that are looking at the issue of DNA hypermethylation. For those of you who aren’t familiar with this field, the promoter regions of certain critical tumor-suppressor genes, such as P16, have been observed on occasion to be hypermethylated in the setting of carcinogenesis, and hypermethylation of a promoter region turns off expression of that gene. In fact, there’s accumulating evidence that one of the ways that tumor-suppressor genes become inactivated in carcinogenesis is by having inappropriate hypermethylation of their promoter regions. Interestingly DNA methylation may be aberrant in a number of ways at the same time. If you look at any human cancer you will see genomic hypomethylation, and if you look at the coding regions of certain genes, you’ll see DNA hypomethylation. But in that same person or that same tumor, you will also at the same time see hypermethylation. We don’t know exactly which is the more critical phenomenon.

To address the second portion of your question, how folate might be involved in hypermethylation, I would point you towards some of the work that’s come out of James’s laboratory at the National Center for Toxicology Research in the USA. They are also very interested in folate in carcinogenesis, and they’ve done several studies where they impose folate and methyl deficiency on animals. Initially there’s hypomethylation, but if you follow these animals over a period, in some genes there seems to be a compensatory increase in methylation. There’s some regulatory mechanism that senses hypomethylation in certain portions of the genome and somehow the cell compensates for that – in fact it overcompensates for that by hypermethylating the same region. So in these studies it may be very important to look at what’s happening over time. So depending upon the particular locus that you’re looking at in the genome, folate depletion can cause either hypomethylation or hypermethylation.

Dr. Heimburger: We’ve been working on two other organs, the cervix and the lung, and we have data that have been submitted for publication using a somewhat similar model – for example, using tissues from lung cancer and adjacent normal tissue and comparing methylation levels within the tissues and also tissue vitamin levels. Chandrika has found that there is the expected correlation between folate levels and methylation levels in the tissues – that is global DNA methylation. But she also found a rather unexpected accumulation of vitamin C in those tissues, and a significant correlation between the accumulated vitamin C and better methylation. Can you think of any mechanism through which an accumulation of vitamin C might protect against hypomethylation? It is clear that folate isn’t the only thing that influences DNA methylation. There are a lot of other factors as well.

Dr. Mason: This is an interesting observation. It’s the first time I’ve heard of it and I can’t immediately think of a mechanism that would explain it. Interestingly, Giuliano’s group in Arizona published a similar study on the cervix recently where they looked at folate levels and DNA methylation and saw the same relation that you spoke about.

Dr. Endres: We have found that in neuroblastoma patients the concentration of cystathionine is increased in liver metastases and it could be that there is vitamin B₆ deficiency. Cystathionine synthase and cystathionase have pyridoxine or pyridoxyl 5-biphosphate as the cofactor. My question is, are there studies where the pyridoxine status has been investigated?
**Dr. Mason:** The issue of vitamin B₆ depletion and enhanced carcinogenesis has not been examined extensively, though Albanes and Schilue collaborated in showing an association between B₆ and pancreatic cancer, which was published recently in the *Journal of the National Cancer Institute*.

**Dr. Endres:** Also, are you aware of investigations concerning hyperhomocysteinemia in patients with colorectal cancer?

**Dr. Mason:** Most of the studies that have looked at the issue of folate and cancer haven’t yet examined the homocysteine issue. Interestingly, in one of the studies we published last year on colonic adenomas, we showed that not only were colonic folate levels lower, even in the face of similar systemic folate levels, but the individuals who harbored adenomas also had a very small but statistically significant increase in homocysteine in their blood. So this suggests that these individuals may have some type of systemic defect in folate metabolism that led not only to a lower level of colonic folate, but also to a slight but detectable increase in serum homocysteine.

**Dr. Riboli:** Maybe I can add that a few months ago, in collaboration with the New York University group, we published the first study on homocysteine levels and colorectal cancer. It was a study based on a cohort of 14,000 women followed up for about 10 years, and we measured homocysteine and folate, and blood samples were taken at baseline. There was a doubling of incidence of colorectal cancer in women in the lowest quartile of homocysteine levels. So what we found fits perfectly with this model. In our study, the association was actually stronger with low levels of homocysteine than with low levels of folate.

**Dr. Mason:** If you look at the literature on folate metabolism, it’s clear that elevations in homocysteine are a very sensitive indicator of early folate depletion. You might be right that because it’s so sensitive it would in some respects be better to look at that rather than at serum levels of folate or red cell folate.

**Dr. Go:** We published a paper recently on a rat folic acid deficiency model in the *Journal of Nutrition and Cancer*. Malignancy occurred only in the liver. We didn’t see any malignancy or adenomas anywhere else at all. My question is, when you look for DNA strand breaks or methylation abnormalities in your model, have you found altered specificity and sensitivity in different organ systems?

**Dr. Mason:** Yes we have. But first, let me remind everyone that I’m not suggesting that folate deficiency by itself is a carcinogen. I think that folate depletion, when it’s present in conjunction with an underlying predisposition of cancer, enhances carcinogenesis. I think it’s fairly clear – primarily from animal studies – that sensitivity to folate depletion varies widely between the organs. I can think of one study in particular that showed that if you put an animal on a folate-depleted diet, there were falling concentrations of folate in various tissues, but even at the end of the study brain levels of folate were entirely normal. They were maintained for around 10 weeks of folate depletion. So the body obviously works very hard to maintain folate levels in the brain and CNS, even in the face of folate depletion throughout the rest of the body. So different tissues clearly have different susceptibilities to folate. I think that’s one of the reasons why the colon might be the organ that most explicitly reflects the relation between folate and cancer; remember the colonic mucosa turns over very rapidly, and any organ that proliferates rapidly has a lot of DNA synthesis going on, with folate integrally involved.