Altered Folate Availability Modifies the Molecular Environment of the Human Colorectum: Implications for Colorectal Carcinogenesis

Petr Protiva1,2,3, Joel B. Mason4, Zhenhua Liu5, Michael E. Hopkins1, Celeste Nelson1, James R. Marshall5, Richard W. Lambrecht3, Swaroop Pendyala1, Levy Kopelovich6, Myungjin Kim7, Steven H. Kleinstein8, Peter W. Laird7, Martin Lipkin9, and Peter R. Holt1

Abstract
Low folate status increases colorectal cancer risk. Paradoxically, overly abundant folate supplementation, which is not uncommon in the United States, may increase risk. The mechanisms of these effects are unknown. We conducted two translational studies to define molecular pathways in the human colon altered either by folate supplementation or by dietary folate depletion (followed by repletion). In the first study, 10 healthy, at-risk volunteers (with documented stable/normal folate intake) received supplemental folic acid (1 mg/d) for 8 weeks. In the second study, 10 similar subjects were admitted to a hospital as inpatients for 12 weeks to study folate depletion induced by a low folate diet. A repletion regimen of folic acid (1 mg/d) was provided for the last 4 of these weeks. Both studies included an 8-week run-in period to ensure stabilized folate levels prior to intervention. We obtained 12 rectosigmoid biopsies (from 4 quadrants of normal-appearing mucosa 10–15 cm from the anal verge) at baseline and at measured intervals in both studies for assessing the primary endpoints: genome-wide gene expression, genomic DNA methylation, promoter methylation (depletion/repletion study only), and p53 DNA strand breaks. Serum and rectosigmoid folate concentrations accurately tracked all changes in folate delivery (P < 0.05). In the first study, gene array analysis revealed that supplementation upregulated multiple inflammation- and immune-related pathways in addition to altering several 1-carbon–related enzymes (P < 0.001). In the second study, folate depletion downregulated genes involved in immune response, inflammation, the cell cycle, and mitochondrial/energy pathways; repletion reversed most of these changes. However, changes in gene expression after repletion in the second study (involving immune response and inflammation) did not reach the levels seen after supplementation in the first study. Neither genomic nor promoter-specific DNA methylation changed during the course of the depletion/repletion protocol, and genomic methylation did not change with supplementation in the first study. p53 DNA strand breaks increased with depletion after 12 weeks. In sum, depletion downregulates, whereas repletion or supplementation upregulates pathways related to inflammation and immune response. These findings provide novel support to the concept that excessive folate supplementation might promote colorectal carcinogenesis by enhancing proinflammatory and immune response pathways. These results indicate that modest changes in folate delivery create substantial changes in the molecular milieu of the human colon. Cancer Prev Res; 4(4): 530–43. ©2011 AACR.
Introduction

Epidemiologic observations and preclinical studies have demonstrated that habitually low intake of folate, or indicators of diminished systemic folate status, are associated with an increased risk of cancer of the colorectum (1–5). However, folate intake at supraphysiologic levels among individuals who already possess an existing focus of neoplastic cells may paradoxically promote cancer development (reviewed in ref. 1). The latter is of particular concern since increased consumption of folate, through the use of supplements and additions to the food stream, has increased mean blood folate levels considerably over the past 2 decades (6).

Little is known about the specific cellular pathways through which either inadequate or overly abundant folate intake modulates the risk of colorectal cancer in humans. Folate is an essential cofactor in DNA methylation and DNA synthesis and repair, aberrations of which are commonly implicated in colorectal carcinogenesis. Rodent studies have demonstrated that a mild depletion of folate and other 1-carbon nutrients alone is sufficient to enhance certain protransformational signaling pathways in the colonic mucosa (7, 8), presumably leading to neoplastic transformation when complemented by other procarcinogenic factors. The mechanistic basis for the procarcinogenic effect of excessive folate intake is also ill-defined, although it has been posited that an existing focus of neoplastic cells responds to increasing folate concentrations by accelerating DNA synthesis and cellular proliferation (9, 10). An alternative explanation for the promotional effect of excess folate comes from the recent secondary analysis of a trial of aspirin and folate in the setting of colorectal polyyps: observations from this study suggest that folate supplementation attenuates the anti-inflammatory effect of aspirin (11).

Elucidating pathways through which folate modulates colorectal carcinogenesis will assist in the development of public health care strategies that are both safe and effective in reducing colorectal cancer risk. Therefore, by monitoring gene expression signatures, DNA methylation patterns, and a representative example of gene-specific DNA strand breaks, we sought to define molecular events that result from folate supplementation and from folate depletion. We examined these issues in 2 cohorts of human subjects who were at a modestly elevated risk of colorectal cancer. Folate supplementation was provided to folate-replete individuals in the first study. In the second study, a period of depletion was first studied, followed by a period in which the depleted subjects underwent repletion with folic acid supplements.

Materials and Methods

Subjects

Eligibility criteria for subjects in both studies included individuals who were 44 to 72 years of age and who had a history of either a sporadic colorectal adenoma or a first-degree relative with a sporadic colorectal cancer or adenoma. Exclusion criteria included a history of cancer other than nonmelanoma skin cancer, of intestinal surgery, inflammatory bowel disease, malabsorption, estrogen/progesterone replacement therapy, supplemental vitamin D or regular use of non–steroidal anti-inflammatory drugs. The 2 clinical protocols including correlative laboratory studies were approved by the Internal Review Board (IRB) of Rockefeller University Hospital and the IRBs of the other collaborating institutions, and informed consent was obtained from all subjects.

Diet and study designs

Research dieticians instructed study subjects to maintain their prestudy self-selected diet for at least 8 weeks prior to each study (the “run-in” period). These diets provided sufficient calories and nutrients to maintain energy balance and contained quantities of calcium and vitamin D that approximate current recommended intakes (Table 1). No subject was taking a multivitamin preparation containing folic acid prior to the run-in period. To facilitate folate depletion for the depletion protocol, subjects were instructed to avoid folate-containing vitamin supplements and fortified ready-to-eat cereals during the run-in phase, since these are the 2 greatest sources of folic acid in the American diet (12). Average dietary intakes during the run-in phase were calculated on the basis of validated 3-day questionnaires and 24-hour recalls which were randomly checked during telephone calls at which time the diet of each volunteer was reviewed. The run-in diets contained a calculated daily mean of 327 μg of folate. All subjects maintained their prestudy weights within 1.5% of the basal value. Each study had a run-in period prior to intervention, as described below (Fig. 1).

Folate supplementation protocol. After the 8-week run-in, subjects were given 1 mg of folic acid orally daily for 8 weeks (Barr Laboratories) while maintaining their run-in diet. The dose of 1 mg was selected as an amount that is commonly used for pharmacologic purposes in clinical settings and clinical trials. Compliance was maximized by selection of motivated volunteers and by close monitoring of all meal and supplement consumption by the inpatient nursing staff and by outpatient nutritionist.

Inpatient folate depletion/repletion protocol. During the 12-week residence in the Metabolic Unit of The Rockefeller University Hospital, all meals were consumed in the Unit. The folate depletion diet consisted of 3 different daily menus that were rotated and prepared specifically to meet each volunteer’s caloric needs. All the diets were analyzed for macronutrient and micronutrient content at Covance Laboratories. Folate content was independently verified by the Mason laboratory using the microtiter plate Lactobacillus casei assay, as previously described (13). The mean folate contents of the 3 rotating diets were 92 ± 6 (SEM) and 73 ± 2 μg/d, as measured by the Mason and Covance laboratories, respectively. The diets of all subjects were supplemented with vitamins and nutrients other than...
folic acid to meet their daily requirements. After subjects consumed the low folate diet for 8 weeks, they were administered 1 mg of folic acid orally daily for 4 weeks (Barr Laboratories) while remaining on the low folate diet.

**Experimental endpoints**

After the 8-week run-in period, subjects underwent a baseline blood sample collection and rectosigmoid biopsies and then were either given 1 mg of folic acid daily for 8 weeks as outpatients (supplementation study) or admitted for an inpatient stay of 12 weeks (10 subjects, depletion/repletion study). Samples for experimental endpoints were collected at baseline, and then again after 4 and 8 weeks in both protocols. In addition, samples were collected at 12 weeks (after 4 weeks of folate repletion) in the depletion protocol. Blood draws were performed via venipuncture. Blood mononuclear cells were isolated by Ficoll gradient centrifugation. Serum, plasma, and mononuclear cell samples were immediately frozen and blood samples for clinical laboratory studies were processed immediately. Flexible proctosigmoidoscopy was performed after a 60-mL tap water enema and approximately 12 biopsies of rectosigmoid mucosa were taken from 4 quadrants at 10 to 15 cm from the anal verge, placed in Nunc CryoTubes, and immediately frozen in liquid nitrogen. Biopsies were taken after close inspection to exclude any mucosal abnormalities. Biopsies were subsequently extracted for RNA (using 2 separate biological duplicates), as well as for DNA.

**DNA extraction**

DNA was extracted from human biopsies using Qiagen DNeasy kit as described by the manufacturer (Qiagen). Frozen mucosal biopsies and mononuclear cells were lysed in DNA extraction buffer and subjected to digestion by proteinase K. DNA was purified and extracted using silica spin columns. DNA was extracted by a conventional phenol-chloroform method and purity was confirmed by the spectrophotometric 260:280 ratio: all samples had a ratio greater than 1.8.

**Rectosigmoid biopsy folate content**

Rectosigmoid mucosal folate concentrations were measured as described previously (14, 15). Three rectosigmoid biopsies from each subject at each time point were combined, rapidly weighed, and tissue folates were immediately extracted in 20 volumes of freshly prepared folate extraction buffer [5 mmol/L β-mercaptoethanol and 0.1 mol/L sodium ascorbate in 0.1 mol/L bis(2-hydroxyethyl)iminotriis (hydroxymethyl) methane (pH = 7.85)] at 95 °C for 20 minutes, which extracts more than 95% of tissue folates. Extracts were then treated with chicken pancreas conjugase to convert

---

**Table 1. Subjects characteristics and dietary intake**

<table>
<thead>
<tr>
<th>Subjects characteristics</th>
<th>Age, y</th>
<th>Weight, kg</th>
<th>Height, cm</th>
<th>Body mass index, Kg/m²</th>
<th>Gender</th>
<th>Ethnicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Inpatient study</td>
<td>Mean</td>
<td>54.0</td>
<td>86.9</td>
<td>176.3</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>9.2</td>
<td>17.6</td>
<td>11.8</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>Outpatient study</td>
<td>Mean</td>
<td>57.6</td>
<td>86.5</td>
<td>171.0</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>7.3</td>
<td>16.7</td>
<td>6.2</td>
<td>5.7</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Daily average outpatient dietary intake</th>
<th>Energy, kcal</th>
<th>Fat, %</th>
<th>CHO, %</th>
<th>Protein, %</th>
<th>Fiber, g</th>
<th>Calcium, mg</th>
<th>Folate, μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inpatient study</td>
<td>Mean 2,635.0</td>
<td>35.1</td>
<td>49.9</td>
<td>15.0</td>
<td>21.0</td>
<td>1,178.0</td>
<td>339.0</td>
</tr>
<tr>
<td></td>
<td>SD 522.0</td>
<td>3.6</td>
<td>1.4</td>
<td>3.0</td>
<td>7.6</td>
<td>706.0</td>
<td>126.0</td>
</tr>
<tr>
<td>Outpatient study</td>
<td>Mean 2,164.0</td>
<td>37.5</td>
<td>46.4</td>
<td>16.7</td>
<td>18.8</td>
<td>685.0</td>
<td>326.0</td>
</tr>
<tr>
<td></td>
<td>SD 342.0</td>
<td>8.2</td>
<td>11.0</td>
<td>5.0</td>
<td>2.7</td>
<td>309.0</td>
<td>69.8</td>
</tr>
</tbody>
</table>

**Risk factors for colon cancer**

| Inpatient study | Family history of colorectal cancer or adenoma—first-degree relative | 8 |
|                | Personal history of adenoma                                          | 2 |
| Outpatient study| Family history of colorectal cancer or adenoma—first-degree relative | 4 |
|                | Personal history of adenoma                                          | 6 |

*Subjects were excluded if they had a history of cancer other than nonmelanoma skin cancer, previous major intestinal surgery, malabsorption, or bleeding disorders, estrogen and/or progesterone replacement, supplemental vitamin D or non-steroidal anti-inflammatory drugs intake, systemic or bowel inflammatory disorder. Mean ± SD for the 10 completed subjects.

*Daily intake calculated for 3-day food records using ESHA food processing database.*
folypolyglutamates to diglutamate derivatives, which was then subjected to the microbiological assay.

Genomic DNA methylation

Genomic DNA (gDNA) methylation was assessed by a validated quantitative LC/MS method as previously described (16). The isotopomers $^{15}$N$_3$ 2'-deoxycytidine and methyl-D$_3$, ring-6-D$_1$ 5-methyl-2'-deoxycytidine (Cambridge Isotope Laboratories) were used as internal standards. DNA methylation status was defined as a percentage: 5-methylcytosine divided by the total of cytosine plus 5-methylcytosine (16).

Promoter methylation

Promoter methylation was analyzed only in the depletion/repletion protocol. Promoter methylation of 432 genes known to be abnormally methylated in human cancers was assessed in DNA extracted from the colorectal biopsies using a promoter methylation bead array. Briefly, a universal bead array system, developed by Illumina, was utilized to examine the methylation of 1,505 CpG sites contained within promoter (and related 5' untranslated region) regions, as described previously (17). These analyses were conducted at the USC Epigenome Center, University of Southern California. The assay procedure is similar to that described for standard SNP (single nucleotide polymorphism) genotyping and gene expression profiling using universal bead arrays (18), except that 4 oligonucleotides, 2 allele-specific oligonucleotides (ASO), and 2 locus-specific oligonucleotides (LSO) are required for each assay site rather than 3. Briefly, bisulfite-treated, biotinylated gDNA was immobilized on paramagnetic beads. Pooled query oligonucleotides were annealed to the gDNA under a controlled hybridization program, and then washed to remove excess or mishybridized oligonucleotides. Hybridized oligonucleotides were then extended and ligated to generate amplifiable templates. Requiring the joining of 2 fragments to create a PCR template in this scheme provided an additional level of locus specificity. It is unlikely that any incorrectly hybridized ASOs and LSOs will be adjacent, and therefore would be able to ligate after ASO extension. A PCR reaction was performed with fluorescently labeled universal PCR primers. The methylation status of an interrogated CpG site was determined by calculating a $\beta$-value, which is defined as the ratio of the fluorescent signal from the methylated allele to the sum of the fluorescent signals of both methylated and unmethylated alleles. The $\beta$-value provides a continuous measure of levels of DNA methylation in samples, ranging from 0 in the case of completely unmethylated sites to 1 in completely methylated sites.

p53 Strand breaks

Exons 6 and 8 of the p53 hypermutable region (exons 5–8) were chosen because previous animal studies have shown that these regions are susceptible to strand breakage due to dietary folate depletion (8, 19). The detection of p53-specific DNA strand breaks were determined by a previously described quantitative PCR method (20). Assay validation was achieved by testing the effect of increasing amounts of template and of restriction digest on product formation. Strand breaks at p53 exon 6 and 8 are reported as a $\Delta$Ct value (Ct p53 exon 6 or 8 – Ct β-actin), with a higher $\Delta$Ct
indicating a lower template integrity. This assay has been validated as a sensitive means of detecting DNA breaks in the human p53 gene (20) but is not entirely specific since abasic sites, bulky adducts or DNA cross-links may inhibit amplification as well and be detected by this method.

RNA extraction for expression analyses

Frozen human biopsies were maintained in liquid nitrogen until total RNA extraction using the TRIzol method (Invitrogen). TRIzol extracted RNA was further purified using Qiagen RNEasy kits (Qiagen Inc.), yielding high quality RNA suitable for microarray analyses. RNA quality was verified by analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies) and RNA was quantified by NanoDrop (NanoDrop Technologies). Tissue or biological duplicates (i.e., biopsies taken from 2 different mucosal areas from a subject at the same time) were used for gene expression endpoints. In addition, 1 technical replicate (RNA from 1 of the 2 tissue or biological replicates) was used for baseline and 8 weeks of outpatient study to generate array data to increase the power of the analyses at these time points. Total RNA (500 ng) was used for in vitro transcription and cRNA amplification and labeling using Ambion’s ILLUMINA kit according to the manufacturer’s instruction. Biotin-labeled cRNA was labeled with fluorescent dye, hybridized onto Sentrix Human Ref-8 24K Expression Array Bead Chip (Illumina), and scanned. More advanced Version 2 of this array was used for the depletion/repletion study.

Quantitative RT-PCR analysis

For reverse transcriptase PCR (RT-PCR), duplicate 1 µg samples of total RNA were used as template for cDNA synthesis using Superscript III First Strand kit (Invitrogen). cDNA was diluted in RT buffer and amounts corresponding to 100 ng of original RNA were used for gene expression by quantitative RT-PCR using TaqMan Gene Expression Assay probes and primers (Applied Biosystems) and the ABI Prism 7900 PCR system at the University of Connecticut Health Center Gene Array Core Facility. Three most upregulated genes (CCL20, LTF, and TCLI) and 3 most downregulated genes (BEST4, PYH, and GCG) were measured by RT-PCR to validate our array data. Additional genes relevant to 1-carbon metabolism [thymidylate synthase (TYMS), dihydrofolate reductase (DHFR)] and inflammation (IL-6 and OLFM4) were also measured. Expression quantification used the delta method. 18S mRNA and/or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) endogenous controls were used. Three independent PCR reactions were used for each sample to calculate results.

Gene array analyses

Expression data were analyzed by Genespring software (Agilent Technologies, Inc.) after normalization. Quality control was performed analyzing gene expression correlation coefficients. Arrays were normalized to 50th percentile per chip and median per gene, expression values below noise level were set to the minimum detection level. Probes with missing values for at least 1 experimental condition were excluded. For the actual analysis, biological duplicate samples were averaged. The differences in gene expression were determined using repeated measures ANOVA or paired t test, multiple hypothesis testing adjustment were made using Benjamini–Hochberg method at a false discovery rate (FDR) of less than 0.05. If no adjustments were made, it is directly stated in the results. Genes differentially expressed following folic acid administration were subjected to Gene Ontology (GO) analysis using the hypergeometric method (http://www.geneontology.org) corrected by Benjamini–Yekutieli method at FDR-q less than 0.05. Subsequently, Gene Set Enrichment Analysis (GSEA) was used. GSEA is a computational method that determines whether an a priori defined set of genes shows statistically significant differences between 2 phenotypes. We ranked gene expression differences between folate intervention time points and baseline to identify gene sets that were significantly enriched after depletion/repletion or supplementation (http://www.broadinstitute.org/gsea). Both curated and computationally derived data sets were used for result interpretation. FDR-q was used to rank the enrichment results.

Statistics

The number of subjects was determined a priori, using sample size estimates derived from alterations in gDNA methylation of the colon observed in a prior pilot trial of folate supplementation (21). Statistical analysis of gene arrays is described above. Biological duplicates were used to generate array data except for the supplementation study, where at the baseline and 8 week time points, technical replicates were added as well. In each study, a nonparametric Repeat Measures Friedman’s Test with Dunn’s Multiple Comparisons Test was used to compare endpoints at different time points. Significance was set at a 2-tailed value of P < 0.05. For analysis of promoter methylation, a repeat measures analysis with post hoc testing was applied to identify those genes that underwent significant changes over the course of the 4 time points, and a Benjamini–Hochberg test was used to adjust for multiple observations (FDR < 0.05).

Results

Subjects

Twenty healthy, folate-replete subjects were enrolled into the 2 studies (10 subjects each), beginning in 2004 and ending in 2006. We enrolled 12 men and 8 women, aged 44 to 72 years, 8 with a history of sporadic colorectal adenoma resection and 12 with a first-degree relative with sporadic colorectal cancer or adenoma (Fig. 1 and Table 1). No subject had a history of multiple adenomas or multiple family members with colorectal cancer. No rectosigmoid adenomas or other macroscopic abnormalities were detected at study sigmoidoscopies. Twelve subjects were Caucasian, 6 African-American, and 2 of mixed racial background (Table 1). The supplementation study was conducted on an outpatient basis. The depletion/repletion
study was conducted in the inpatient Metabolic Unit of The Rockefeller University Hospital. All 20 subjects successfully completed the 8-week run-in and experimental periods without development of anemia. None of the subjects complained of significant adverse effects. On the basis of pill counts, subjects consumed an average of 99% of their prescribed supplements.

**Folate supplementation study**
Serum and rectosigmoid folate concentrations each increased by 50% to 80% during the 8 weeks of folic acid supplementation compared with baseline (\(P < 0.05\); Fig. 2). No statistically significant changes in RBC folate or homocysteine levels occurred between baseline and the end of the study although numerically, an 11% decrease in homocysteine was observed (mean decrease from 8.54 to 7.6 \(\mu\)mol/L) and RBC folate increased from a mean of 472 ng/mL to 498 ng/mL (Fig. 2).

**Folate depletion/repletion study**
Mean serum and rectosigmoid folate concentrations each decreased by 50% to 80% during the 8 weeks of folate depletion compared with baseline (\(P < 0.01\) and \(P < 0.05\), respectively; Fig. 2). In addition, a nonsignificant decline of 16% was observed in RBC folate after 8 weeks of depletion. In response to folate depletion, serum homocysteine levels increased (by 13%; \(P < 0.05\)). The folate repletion was also effective; although, most indicators of folate status fell just short of reverting to baseline levels, significant increases in serum and colonic folate, and a decrease in serum homocysteine levels were each observed after 8 weeks of depletion: significant increases in serum and colonic folate, and a decrease in serum homocysteine levels were each observed (all \(P < 0.05\)). No statistically significant increase in RBC folate levels occurred between the end of the depletion phase and after supplementation (increase of 2%, \(P = 0.33\)). No statistically significant differences in serum or colonic folate or serum homocysteine were found between baseline values and those observed at the end of the study.

**p53 Strand breaks**
DNA strand breaks contained within exon 6 and exon 8 of the p53 gene were independently examined in rectosigmoid biopsies. In the outpatient supplementation study, no changes in strand breaks were observed in the colon over the course of the study. In the inpatient depletion/repletion study, a gradual increase in exon 6 strand breaks of p53 gene were observed over the course of the entire study, although this did not achieve statistical significance until the 12-week time point. Similarly, an increasing trend was observed in exon 8 strand breaks although the mean values never achieved statistical significance (see Supplementary Fig.).

**Genomic DNA methylation**
Genomic DNA methylation was studied at 4 and 8 weeks following dietary folate depletion, at 12 weeks following 4 weeks of folic acid repletion, as well as during outpatient supplementation. No statistically significant changes were detected.

**Promoter methylation**
Due to the limited amount of DNA extracted from biopsies, promoter methylation could not be studied in the outpatient supplementation protocol. In 3 of 10 subjects from the inpatient depletion/repletion protocol, complete methylation data were available for all 4 time points of the study (inadequate quantities of colonic DNA were available at 1 or more time points for the remaining subjects). After adjusting for multiple observations by Benjamini–Hochberg test, no promoter was found to be differentially methylated to a significant degree. These data suggest that short-term modest changes in folate status do not result in significant changes in promoter methylation in the colonic mucosa.

**Gene array analysis**
Quality assurance analysis was performed by analyzing gene expression correlations between biological and/or technical duplicates. In the supplementation study, 2 subjects (subject 23 and 24) showed a correlation coefficient of 0.95 or less and thus were excluded from the gene array analysis. All samples from the inpatient depletion/repletion study showed a correlation coefficient of 0.99 or greater, and so all array data from this study were included in the analysis. Unsupervised hierarchical condition clustering analysis showed that samples from the 10 depletion/repletion and 8 supplementation study subjects were divided into 18 distinct clusters according to their similarity measures. Each of these clusters corresponded to samples from a single subject suggesting that gene expression for the 24,000 interrogated genes maintained similarity within the rectosigmoid mucosa during the length of the study. Similar data were observed in a previous study (22).

**Folic acid supplementation.** Using Repeat Measures ANOVA test and Benjamini–Hochberg Multiple Testing Correction, no genes were identified as being differentially expressed compared with the baseline. Unadjusted test showed that 1,001 genes were differentially expressed at \(P < 0.05\). At both 4 weeks and 8 weeks of supplementation, 306 genes were upregulated and 308 genes were downregulated (Table 2). Most upregulated GO categories were immune system process (\(P < 0.001\)) and immune response (\(P = 0.001\)), and most downregulated categories were regulation of transcription (\(P = 0.001\)) and regulation of nucleotide and nucleic acid metabolic process (\(P = 0.001\), all unadjusted analyses). We then compared baseline with 8 weeks of supplementation by paired t test using additional technical replicates. A total of 2,073 genes were differentially expressed at \(P < 0.05\) (unadjusted paired t test; Table 2). More than 80% of genes down- or upregulated at 8 weeks of supplementation showed the same changes at 4 weeks. A total of 642 upregulated genes were consistently related to immune and inflammatory processes such as immune system process and immune.
response ($P < 0.001$), inflammatory response ($P = 0.001$), chemokine activity ($P = 0.004$), and cytokine receptor binding ($P < 0.001$, all adjusted analyses; Table 3). A total of 1,431 downregulated genes were related to cellular metabolic processes ($P < 0.001$), nucleus ($P < 0.001$), regulation of nucleotide and nucleic acid metabolic process ($P < 0.001$), cell cycle ($P < 0.001$), mitosis ($P = 0.002$, all adjusted analyses); all significant genes are listed in the Supplementary Table S1.

Analysis of the methionine (‘1-carbon’) metabolism pathway showed significant downregulation during folic acid supplementation ($P = 0.02$). The supplementation
regimen modulated expression of several enzymes in the colonic mucosa important in nucleotide synthesis and biological methylation (Fig. 3). Additional downregulated genes linked to 1-carbon metabolism that are not shown in the figure include the folate receptor 1 (FOLR1), and gamma glutamyl hydrolase (conjugase, GGH).

In addition to the statistical analysis, we analyzed the top 1% or 5% regulated genes in our supplementation trial and we observed similar results to those from unadjusted ANOVA or t tests (Supplementary Table S2).

**Depletion/repletion study.** Using Repeat Measures ANOVA test and Benjamini Hochberg Multiple Testing Correction, 1,585 genes were identified as being differentially expressed compared with the baseline (Table 2), all significant genes are listed in Supplement Table S3. Adjusted GO analysis showed that genes that were downregulated at 4 and 8 weeks were related to cellular metabolic processes ($P = 0.029$), nucleus ($P = 0.048$), immune system process ($P = 0.069$), and immune response ($P = 0.076$). There were no significantly enriched upregulated GO categories in adjusted analysis; but, unadjusted analysis revealed that genes upregulated at 4 and 8 weeks of depletion were most significantly related to γ-aminobutyric acid (GABA) signaling and receptor activity ($P = 0.001$). Next, we examined changes in gene expression after folic acid repletion and observed that repletion phase of the study generally produced reciprocal changes to those observed with depletion, although exceptions did exist (Fig. 4, bottom). There were 281 genes whose expression was downregulated by folate depletion and upregulated by folate repletion (vs. 8-week depletion time point). Adjusted analysis showed that immune response ($P = 0.027$) and immune system process ($P = 0.027$) GO categories were enriched. There were 689 genes upregulated by folate depletion and redownregulated after repletion. Adjusted analysis showed no significant GO categories but top-enriched GO groups in unadjusted analysis were again GABA receptor signaling and activity ($P = 0.001$). Because of the folate intervention, the methionine ("1-carbon") metabolic pathway was analyzed; it was not significantly enriched during folate depletion/repletion.

We observed that changes in immune, inflammatory, and other pathways were associated with changes in folate status. To confirm these findings and to directly compare changes across both studies, we used GSEA. Figure 4 shows GO categories most significantly and consistently modu-

---

**Table 2. Gene expression changes detected by statistical methods**

<table>
<thead>
<tr>
<th>Folate status</th>
<th>Depletion</th>
<th>Repletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time point, wk</td>
<td>4 8 12 (vs. 8)</td>
<td></td>
</tr>
<tr>
<td>No. of Genes</td>
<td>1,585</td>
<td></td>
</tr>
<tr>
<td>Upregulated</td>
<td>806 999 365</td>
<td></td>
</tr>
<tr>
<td>Downregulated</td>
<td>779 584 1,209</td>
<td></td>
</tr>
</tbody>
</table>

**Repeated measures ANOVA**

<table>
<thead>
<tr>
<th>Folate status</th>
<th>Supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time point, wk</td>
<td>4 8</td>
</tr>
<tr>
<td>No. of Genes</td>
<td>1,001</td>
</tr>
<tr>
<td>Upregulated</td>
<td>522 477</td>
</tr>
<tr>
<td>Downregulated</td>
<td>479 524</td>
</tr>
</tbody>
</table>

**Repeated measures ANOVA**

| No. of Genes | 2,073 |
| Upregulated | 642 |
| Downregulated | 1,431 |

**Paired t test baseline vs. 8-wk supplementation**

| No. of Genes | 2,073 |
| Upregulated | 642 |
| Downregulated | 1,431 |

- Adjusted by Benjamini–Hochberg Multiple Correction Test.
- Modulated at both 4 and 8 weeks.
- Unadjusted analysis.

---

**Table 3. List of genes involved in selected inflammatory and immune-related GO categories that were significantly upregulated following folic acid supplementation**

**Cytokine and chemokine activity**

CCL14, CCL19, CCL20, CCL21, CCL22, CCL23, CSH2, CXCL1, CXCL13, EB13, ECGF1, IL-17F, IL-27, IL-29, IL-6, LTB, PF4V1, TNF, TNFSF11

**Immune response**

APS, BATF, BF, C3, C4A, CCL14, CCL20, CCL21, CCL22, CCR6, CCR7, CD1C, CD22, CD72, CFHL1, CRFL1, CTLA4, DETA5, DEFA6, EB13, EDG6, FADD, FCGR3A, G1P2, G1P3, GPSM3, ICOS, IKBKE, IL-12RB1, IL-6, IL-8ST, INDO, ISGF3G, KIR2DS2, LTB, LTF, MHC2TA, MX1, MX2, OAS2, PF4V1, PFC, PSMB9, RNF125, SCAP1, TLR10, TNF, TNFRSF9, TNFRSF11, TNFSF13, UBD

**Inflammatory response**

ALOX5AP, BF, C3, C4A, CCL19, CCL20, CCL21, CCL22, CCL23, CCL23, CCR7, CFHL1, CXCL1, CXCL13, FN1, IL-17F, IL-6, INS, NCR3, PFC, SERPINF2, TLR10
lated by folate status were indeed related to immune and inflammatory pathways. Individual genes contributing to enrichment of immune system process and immune response pathways are listed in the Supplementary Table S4. Of interest is that another group of genes regulated by folate status is related to neuronal channel activities that also includes GABA activity. Full list of significantly enriched pathways across both studies is listed in the Supplementary Table S5.

Quantitative RT-PCR validation of differentially expressed genes
To validate array data, the 3 most upregulated genes (LTF, CCL20, and TL1C) and the 3 most downregulated genes (BEST4, GCG, and PYY) were selected from the 8 subjects analyzed by gene arrays from the folic acid supplementation study for RT-PCR from a list of significant genes identified by paired t test. To validate the changes in inflammatory genes and genes involved in 1-carbon metabolism (Fig. 3), OLFM4, IL-6, DHFR, and TYMS were also measured. RT-PCR results confirmed upregulation of TL1C ($P = 0.04$) and upregulation of inflammatory markers OLFM4, CCL20, LTF, and IL-6 by RT-PCR ($P = 0.02$, $P < 0.01$, $P = 0.01$, and $P = 0.06$, respectively) and downregulation of BEST4, GCG, and PYY ($P < 0.01$ for all 3 genes). Downregulation was also confirmed for DHFR and TYMS, 2 genes involved in 1-carbon metabolism ($P < 0.01$ for both genes; Fig. 5).

Discussion
This study defined alterations that occurred in the human colon in gene expression and other relevant molecular events in response to: (i) 2 months of dietary folate depletion and (ii) 2 months of folic acid supplementation. Subjects who by history were at modestly increased risk of colon cancer were studied since the underlying concept was to define which cancer-relevant pathways are altered by varying levels of folate intake in individuals whose colons are susceptible to neoplastic transformation. The modest
changes in colonic folate status that were induced by each of the 2 protocols produced remarkable shifts in gene expression. The pattern of changes observed with supplementation are particularly informative since they appear to provide a mechanistic explanation for recent clinical observations that indicate that folic acid supplements may contribute to a proinflammatory environment in the colon.

Although folate depletion is difficult to achieve in the present era of mandatory folic acid fortification, the first study successfully induced a significant degree of depletion, as evidenced by substantial declines in serum and colonic folate, and an increase in serum homocysteine (Fig. 2). In fact, homocysteine rises when intracellular folate concentrations can no longer adequately support biological remethylation (23), so the degree of depletion achieved in the study is indicative of a true intracellular depletion of the vitamin. Nevertheless, the degree of depletion at the end of the study was rather modest: since after depletion,
the mean serum folate concentration (5.8 ng/mL) did not fall below the threshold that is conventionally considered to define the lower limit of normality (i.e., 5 ng/mL). This is clinically relevant since enhancement of colorectal cancer risk due to folate inadequacy often occurs in segments of the population whose low folate status is at the lower end of the normal range rather than in the zone of frank deficiency (24). Furthermore, repletion of folate also was successful since indicators of folate status, both systemic and colonic, returned nearly to baseline status. RBC folate status did not similarly increase to a significant degree presumably since the half-life of red cell folate parallels the half-life of those cells. These data agree with previous studies which have demonstrated that serum folate is a more accurate proxy measure of human colonic folate status than RBC folate (14). In the second study, in which supplements were administered to individuals who were already folate-replete, these changes were upregulated to an even greater degree (Supplementary Tables S1–4). Therefore, even though repleting folate-depleted subjects with folic acid supplements is physiologically distinct from supplementing folate-replete subjects, the effects on the expression of proinflammatory and immune-related genes are very similar. Consistent with these results is a recent human study that showed that administration of 1.2 mg folic acid for 12 weeks increases serum proteins involved in the regulation and activation of immune function and the complement cascade (25). Collectively, these observations may constitute the mechanistic basis for recent observations emerging from the human Aspirin/Folate Polyp Prevention trial, in which folic acid administration was observed to antagonize the suppressive effects of aspirin on circulating inflammatory markers (11). A similar effect has been observed in an animal model of hepatic injury, in which supraphysiologic
levels of dietary folic acid led to enhanced inflammation and tissue damage (26).

Our data also show that neuronal receptor pathways were also linked to folate status. Expression of genes, such as GABA receptors, or other G-protein–coupled receptors, such as taste transduction receptors, were increased during folate depletion and reciprocally decreased during folate repletion. The potential association between these genes and colon carcinogenesis is unclear, but GABA may be overexpressed in colon cancer and GABA receptor signaling was linked to metastatic behavior of colon cancer cells in experimental models (27).

Metabolism of 1-carbon is complex, making it difficult to accurately predict the net metabolic effects of the changes in gene expression in 1-carbon enzymes that we observed in the present study. Some of these changes warrant speculation, however. Thymidylate synthase expression was suppressed by supplementation (confirmed by qRT-PCR; Fig. 5), which could diminish thymidylate availability for DNA synthesis and thereby enhance uracil incorporation into DNA, a potentially mutagenic event. Thus, our observations are consistent with a report showing that subjects receiving 5 mg of folic acid per day developed increased levels of uracil in colonic mucosa (28).

Our results also suggest that altered expression in 1-carbon enzymes may represent a protective mechanism against an oversupply of folate cofactor. By downregulating DHF, folic acid would be less capable of entering into the 1-carbon metabolic network (Fig. 3). Moreover, the terminal enzymes for 2 critical functions of 1-carbon metabolism, thymidylate synthesis and biological methylation of DNA, were both downregulated, an effect which might limit the synthesis of end products in the face of excess cofactor. This result is consistent with results of Basten and colleagues, who observed that supplemental folic acid administered to healthy volunteers in a dose and duration similar to those in our study diminished DNA excision repair (29). They postulated that the surfeit of nucleotides provided for by excess folic acid might downregulate pathways related to DNA repair, a concept that was borne out by our observation that supplementation led to a downregulation of the pathways integral to both purine and thymidine synthesis. It is feasible that these changes also may alter cell-cycle dynamics since we observed significant downregulation of genes that are involved in regulation of cell cycle and DNA replication including cyclins, cyclin-dependent kinases, and proliferating cell nuclear antigen (Supplementary Table S1).

We examined gDNA methylation in both studies and found no significant changes. The observation that the colon is relatively resistant to changes in genomic methylation due to altered folate status has also been noted in animal studies (30) as well as in some (31), but not all (32), clinical trials.

Probably more relevant to mechanistic issues than genomic methylation were the observations pertaining to loci-specific methylation. We found no significant changes in promoter methylation in response to folate depletion or in the subsequent phase of repletion. Although promoter methylation has been modified with folate depletion in some cell culture studies (33), the severity of deficiency in such preclinical models is rather profound and it is entirely feasible that the magnitude of depletion in clinical situations is not robust enough to alter promoter methylation. Another caveat is that small shifts in methylation (<5%) may not have been reliably detected by the technology used in the present study. A case–control study that previously explored this issue suggested that low folate intake may be related to hypermethylation of the p16 gene (34), but our present study is the first that has directly tested whether folate depletion in humans alters promoter methylation in the colon. The fact that many substantial shifts in gene expression were observed in the depletion/repletion protocol in the absence of changes in gene methylation suggests that the alterations in expression, as well as the changes in DNA strand breaks, were mediated by processes other than changes in promoter methylation. This conclusion is consistent with a recent review of the topic, which proposed that other molecular anomalies that arise in the setting of folate depletion, such as impaired DNA synthesis and repair, are the primary drivers of enhanced carcinogenesis (35). Our observations regarding gene-specific methylation are nevertheless limited to the folate depletion/repletion protocol, by the number of subjects with a complete set of samples, by the sensitivity of the detection method, and by the marked stringency of the multiple comparisons test we imposed on our data, and so we cannot exclude the possibility that some modest changes in promoter methylation occurred that went undetected.

We also were not able to test whether folic acid supplementation can induce a change in promoter methylation. Recently, a cross-sectional examination of samples from the Aspirin-Folate PolyPhl Trial identified a positive relationship between RBC folate—an integrated measure of long-term folate intake—and the promoter methylation of 2 cancer relevant genes in normal-appearing colorectal mucosa (36). This study used a more sensitive pyrosequencing method to detect promoter methylation than that used in the present study. Thus, it is conceivable that proinflammatory immune changes induced by short-term folate supplementation might in the long term result in promoter silencing of genes important in colorectal carcinogenesis.

We also assessed DNA strand breaks in 2 exonic loci within p53 in colonic tissue. Whereas supplementation produced no apparent changes (data not shown), a significant increase in exon 6 breaks occurred by week 12 of the depletion study; strand breaks in exon 8 followed a nearly identical course but never reached statistical significance. This finding is consistent with an earlier study in rodents, where instability of the so-called “hypermutable region” of p53 was noted to increase progressively with increasing severity of folate depletion (19). Of interest, p53 strand breaks were one molecular marker that did not reverse at all with repletion; indeed, it continued to increase numerically during the repletion phase, suggesting that the effect is either irreversible or that it takes longer than...
4 weeks to return to its former state. The ramifications of single- and double-stranded DNA breaks in the exons of p53 are not well understood. On one hand, they may be a marker of p53 instability and indicate an increased risk of mutation, which is consistent with the very high rate of mutations found at exons 6 and 8 (37). On the other hand, base excision DNA repair transiently creates strand breaks (38), and therefore higher levels of strand breaks may indicate exceptionally robust and effective DNA repair activity. Consistent with the latter explanation was our observation that expression of 2 genes in the DNA ligase IV complex, XRCC4 and LIG4, increased significantly after 8 weeks of depletion (Supplementary Table S3), although changes in DNA repair pathways overall fell short of statistical significance.

In conclusion, the 2 studies we report here demonstrate that mild dietary folate depletion over 8 weeks decreases the expression of genes involved in proinflammatory and immune-related pathways and that repletion of depleted individuals or supplementation of replete individuals produces changes in expression of these pathways that are reciprocal to those observed with depletion. These findings imply that if an overabundance of folate intake truly promotes progression of colon carcinogenesis (a phenomenon that remains controversial at present), it might do so by enhancing proinflammatory and immune-related changes in the colorectal mucosa. Since altered profiles of gene expression occurred in the absence of significant changes in promoter methylation in our study, other mechanisms that would mediate changes in expression likely are operative. Perhaps most importantly, these results underscore the fact that modest changes in folate delivery create substantial changes in the molecular milieu of the human colon.

Disclosure of Potential Conflicts of Interest

Peter R. Holt was formerly a senior scientist at Strang Cancer Prevention Center, New York.

Grant Support

The study was supported by the GCRC and 5UL1RR024143 from the NCRR and the NIH Roadmap for Medical Research to Rockefeller University, NIH grant N01-CN35111 (PI: JM), CA-29502-24 (PI: PP), Carol and Ray Neag Colon Cancer Prevention Program research funds at University of Connecticut Health Center to P. Protiva, and K05 CA100898 (J.B. Mason).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 28, 2010; revised December 16, 2010; accepted January 6, 2011; published OnlineFirst February 14, 2011.

References

Cancer Prevention Research

Altered Folate Availability Modifies the Molecular Environment of the Human Colorectum: Implications for Colorectal Carcinogenesis

Petr Protiva, Joel B. Mason, Zhenhua Liu, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-10-0143

Cited articles
This article cites 35 articles, 16 of which you can access for free at:
http://cancerpreventionresearch.aacrjournals.org/content/4/4/530.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/4/4/530.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.