

Short Communication

Colonic Mucosal Concentrations of Folate Are Accurately Predicted by Blood Measurements of Folate Status among Individuals Ingesting Physiologic Quantities of Folate¹

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Abstract

Folate status is inversely related to the risk of colorectal cancer. Whether conventional blood measurements of folate status accurately reflect folate concentrations in the colorectal mucosa has been a controversial topic. This is an important issue because accurate measures of folate status in the colorectal mucosa are important for ascertaining the risk of colorectal cancer in epidemiological studies and for determining the effects of folate supplementation in clinical trials. We examined whether conventional blood measurements of folate and a more sensitive, inverse indicator of systemic folate status, serum homocysteine, accurately reflect folate concentrations in human colonic mucosa obtained by endoscopic biopsy. Study subjects ($n = 20$) were participants in a randomized trial that investigated the effect of folate supplementation (5 mg daily for 1 year) on provisional molecular markers of colon cancer. Blood samples and biopsies of normal rectosigmoid mucosa were obtained at baseline, at 6 months, and at 1 year. Serum, RBC, and colonic mucosal folate and serum homocysteine concentrations were determined. Colonic mucosal folate concentrations correlated directly with

serum folate concentrations at each time point ($r = 0.572-0.845$; $P < 0.015$) and with RBC folate concentrations at 6 months and 1 year ($r = 0.747-0.771$; $P < 0.001$). Colonic mucosal folate concentrations correlated inversely with serum homocysteine concentrations at each time point ($r = -0.622-0.666$; $P < 0.008$). Systemic measures of folate status did not correlate with colonic mucosal folate concentrations among individuals receiving supplemental folate. Our observations indicate that colonic mucosal concentrations of folate may be predicted accurately by blood measurements of folate status only among individuals not ingesting supraphysiological quantities of folate.

Introduction

Diminished folate status, assessed by either dietary intake or measurement of blood folate concentrations, has been associated with an increased risk of colorectal cancer and its precursor, adenoma (1, 2). However, the relationship between blood concentrations of folate and colorectal cancer risk is less consistent than that observed between dietary folate intake and colorectal cancer risk (1, 2). The relative insensitivity of blood measurements of folate in predicting colorectal cancer risk may be related to observations indicating that mild folate depletion, rather than the development of overt systemic folate deficiency, is a sufficient condition to enhance the risk of colorectal cancer (1, 2). Also, the effect of folate on the colorectal mucosa may not be derived entirely from blood folate, because the colorectal mucosa is exposed to intestinal luminal folate that is synthesized by intestinal microflora or which has escaped small intestinal absorption (3). There is also some suggestion that a modest reduction in systemic folate status in subjects with colorectal neoplasia may not be apparent with conventional blood measurements of folate. Rather, a more sensitive indicator of cellular folate depletion, such as serum or plasma homocysteine (4), may be necessary to demonstrate this degree of diminished folate status (5). Furthermore, it has also been suggested that localized folate depletion in the colorectal mucosa, in the absence of systemic folate deficiency, may predispose the colorectal mucosa to neoplastic transformation (5).

Whether blood concentrations of folate accurately reflect concentrations in the colonic mucosa has been a controversial topic. Studies in which rodents were fed different dietary amounts of folate have shown that colonic mucosal folate concentrations are significantly correlated with dietary intake and blood folate concentrations (6, 7). In one human study ($n = 30$; Ref. 5), colonic mucosal folate concentrations measured in endoscopic biopsy samples correlated directly with serum and RBC folate concentrations ($r = 0.62$; $P < 0.001$; and $r = 0.46$; $P = 0.013$, respectively) and inversely with serum homocysteine ($r = -0.72$; $P < 0.001$), a sensitive, inverse, systemic measure of cellular folate depletion (4). In contrast, two other

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human studies ($n = 22\text{--}27$) demonstrated no significant correlations between folate concentrations in isolated colonic epithelial cells and serum and RBC folate measurements (8, 9). Thus, there exists a concern that systemic folate indices might not accurately reflect folate concentrations in the colorectal mucosa.

This study investigated whether conventional blood (serum and RBC) measurements of folate and serum homocysteine levels accurately reflect folate concentrations in the colonic mucosa obtained from endoscopic biopsy in subjects who were participants in a randomized trial that investigated the effect of folate supplementation (5 mg daily) on molecular markers in the colon (10). Some of the data from the baseline state were previously reported by us (5). However, the present study adds a very important perspective to our previous observations, because it followed subjects prospectively over time, and because the inclusion of subjects receiving folate supplementation enabled us to examine whether such correlations continued to exist under conditions where subjects were receiving supra-physiological levels of folate.

Materials and Methods

Study subjects were participants in a randomized trial that investigated the effect of folate supplementation (5 mg daily) on genomic DNA methylation and *p53* DNA strand breaks in the colon (10). The detailed protocol and the main study results have been published: both of these provisional biomarkers of colon cancer underwent accelerated improvement at 6 months with folate supplementation compared with placebo (10). Folate supplementation also significantly increased serum (by 2.7–4.4 times), RBC (by 1.9–2.0 times), and colonic mucosal (by 2.2–3.6 times) folate concentrations from the baseline values at 6 months and 1 year (10). In contrast, serum, RBC, and colonic mucosal folate concentrations did not significantly change over time in the placebo group (10). Folic acid pills as well as identically appearing placebo pills were manufactured and supplied by Lederle, Inc. (Pearl River, NY). An independent analysis of the study pills by Hoffman-La Roche (Nutley, NJ) showed that the average folic acid content was 5.47 mg in the folic acid pills and 0 mg in the placebo pills. The protocol was reviewed and approved by the institutional investigational review boards of the Jean Mayer United States Department of Agriculture Human Nutrition Research Center on Aging at Tufts University and the New England Medical Center (Boston, MA). All patients and investigators remained blinded to the treatment assignment during the study period.

Twenty subjects over 18 years of age with histologically confirmed colonic adenomas were randomized to receive either 5 mg folate/day ($n = 9$) or placebo ($n = 11$) for 1 year subsequent to polypectomy after satisfying the specific inclusion and exclusion criteria as published previously (10). At baseline, clinical and laboratory characteristics were not significantly different between the two groups. All subjects underwent colonoscopy at baseline and at 1 year and sigmoidoscopy at 6 months. All patients were prepared for colonoscopy by colonic lavage solution (GoLYTELY; Braintree Laboratories, Inc., Braintree, MA) administered p.o. Colonoscopy was performed in the standard fashion, and all identifiable polyps were removed and processed for routine histology.

At the time of each colonoscopic and sigmoidoscopic examination, three biopsies of normal-appearing mucosa at the rectosigmoid junction (15 cm from the anal verge as measured by the distance of the scope inserted into the colon), at least 5 cm away from any polyp or other mucosal abnormality, were

performed with standard biopsy forceps. These biopsies were immediately placed in a foil packet, immersed in liquid nitrogen, and then stored at -70°C for subsequent analysis of colonic mucosal folate concentrations. After colonoscopy was completed, venous blood was drawn from each subject (in a fasting state) for measurement of serum and RBC folate and serum homocysteine concentrations. Blood for measuring serum folate and homocysteine concentrations was collected into vacuum blood collection tubes and centrifuged at $800 \times g$ for 10 min at 4°C , and serum was stored at -70°C until assayed. The aliquot of serum for the folate analysis was stored in 0.5% sodium ascorbate, which is known to retard folate degradation in storage (11). An aliquot of whole blood, used to measure the RBC folate concentration, was stored in 1% ascorbic acid at -70°C for the same reason.

All assays for serum and RBC folate and serum homocysteine were performed within 2 months of collection. Colonic mucosal folate was extracted from three rectosigmoid mucosal biopsy samples within 1 week of collection and stored at -70°C , and its concentration was determined within 2 months of extraction. Previously, it has been shown that serum and RBC folate and extracted folate from tissues are stable for 12 months when stored at or below -20°C with ascorbic acid (11–13).

RBC folate concentrations were determined by radioassay (MAGIC kit; Ciba-Corning Magnetic Immunochemistries, Medfield, MA). Serum folate was measured by a standard microbiological microtiter plate assay using *Lactobacillus casei* (13). Colonic mucosal folate concentrations were also measured by the microbiological assay, using a previously described method for the determination of tissue folates (14). Three rectosigmoid biopsies from each subject at each time point were combined for folate extraction. A rapid determination of biopsy weight was followed immediately by extraction of tissue folates in 20 volumes of freshly prepared folate extraction buffer [5 mM β -mercaptoethanol and 0.1 M sodium ascorbate in 0.1 M bis(2-hydroxyethyl)imino-tris(hydroxymethyl) methane (pH 7.85)] at 95°C for 20 min. It has been shown previously that more than 95% of tissue folates can be extracted using this technique (15–17). Extracts were then treated with chicken pancreas conjugase to convert all of the folylpolyglutamates to their corresponding diglutamate derivatives (18), thereby enabling the extracts to be subjected to the microbiological assay. Prior studies have shown that a single treatment of tissue folates with chicken pancreas conjugase is sufficient to convert all of the folylpolyglutamates to diglutamates (13, 19). Total serum homocysteine was measured by high-performance liquid chromatography according to the fluorometric method of Vester and Rasmussen (20). All laboratory assays were performed in a blinded fashion.

Blinded replicate quality-control phantom samples from male volunteers aged 50–69 years were placed toward the beginning and the end of each bath. Intra-assay coefficients of variation for folate and homocysteine were 10 and 5%, respectively. Inter-assay coefficients of variation for folate and homocysteine were 11 and 3%, respectively. Previously published studies are associated with the coefficient of variation for this folate assay of $\sim 11\%$ (11–13).

All variables were determined to be normally distributed by a graphic analysis. Linear regression was used to assess the correlation between variables. All significance tests were two-sided and were considered to be statistically significant if the observed significance level (P) was < 0.05 . Statistical analyses were performed by using SYSTAT 5 for Macintosh (Systat, Evanston, IL).

Table 1 Correlations between colonic mucosal concentrations of folate and serum and RBC folate and serum homocysteine concentrations

| | Colonic mucosal concentrations of folate | | |
|-----------------------------------|------------------------------------------|-------------------------|--------|
| | N | Correlation coefficient | P |
| Baseline | 20 | | |
| Serum folate concentrations | | 0.662 | 0.001 |
| RBC folate concentrations | | 0.385 | 0.309 |
| Serum homocysteine concentrations | | -0.622 | 0.003 |
| 6 mo | 18 | | |
| Serum folate concentrations | | 0.572 | 0.013 |
| RBC folate concentrations | | 0.771 | <0.001 |
| Serum homocysteine concentrations | | -0.666 | 0.003 |
| 1 yr | 17 | | |
| Serum folate concentrations | | 0.845 | <0.001 |
| RBC folate concentrations | | 0.747 | 0.001 |
| Serum homocysteine concentrations | | -0.642 | 0.007 |

Results

Twenty subjects were randomized to receive either 5 mg folate/day ($n = 9$) or placebo ($n = 11$) for 1 year. Three subjects (one from the treatment group and two from the placebo group) dropped out of the study. One subject in the placebo group dropped out shortly after recruitment, whereas two patients, one from each group, had to drop out because of myocardial infarcts after 4 and 11 months of follow-up, respectively. Therefore, data from 20, 18, and 17 subjects were evaluated at baseline, 6-month and 1-year follow-ups, respectively. As published previously, folate supplementation, at a dose of 5 mg per day, significantly increased both systemic and colonic mucosal folate concentrations, whereas these parameters did not change significantly during the study period in the placebo group (10).

As shown in Table 1, colonic mucosal concentrations of folate correlated directly with serum folate concentrations at each time point ($r = 0.572$ – 0.845 ; $P < 0.015$). Colonic mucosal concentrations of folate correlated directly with RBC folate concentrations at 6 months and at 1 year ($r = 0.747$ – 0.771 ; $P < 0.001$) but not at baseline (Table 1). Serum homocysteine concentrations correlated inversely with colonic mucosal folate concentrations at each time point ($r = -0.622$ – 0.666 ; $P < 0.008$; Table 1). Fig. 1 demonstrates all values of serum and RBC folate and serum homocysteine concentrations from the three time points plotted against colonic mucosal folate concentrations.

To determine whether blood measurements of folate in conventional physiological ranges and supraphysiological ranges resulting from folate supplementation correlate with colonic mucosal folate concentrations, correlations between blood and colonic mucosal folate concentrations were determined separately for those not receiving folate and those receiving folate supplementation at each time point. As shown in Table 2, serum folate concentrations in physiological ranges correlated well with colonic mucosal concentrations of folate at each time point ($r = 0.662$ – 0.793 ; $P < 0.02$). In contrast, supraphysiological levels of serum folate did not correlate well with colonic mucosal folate concentrations at each time point (Table 2). RBC folate concentrations in physiological ranges also correlated well with colonic mucosal concentrations of folate at each time point ($r = 0.666$ – 0.683 ; $P < 0.05$) except at baseline ($r = 0.385$; $P = 0.309$), whereas no significant association was observed between these two parameters in supraphysiological ranges of RBC folate at each time point

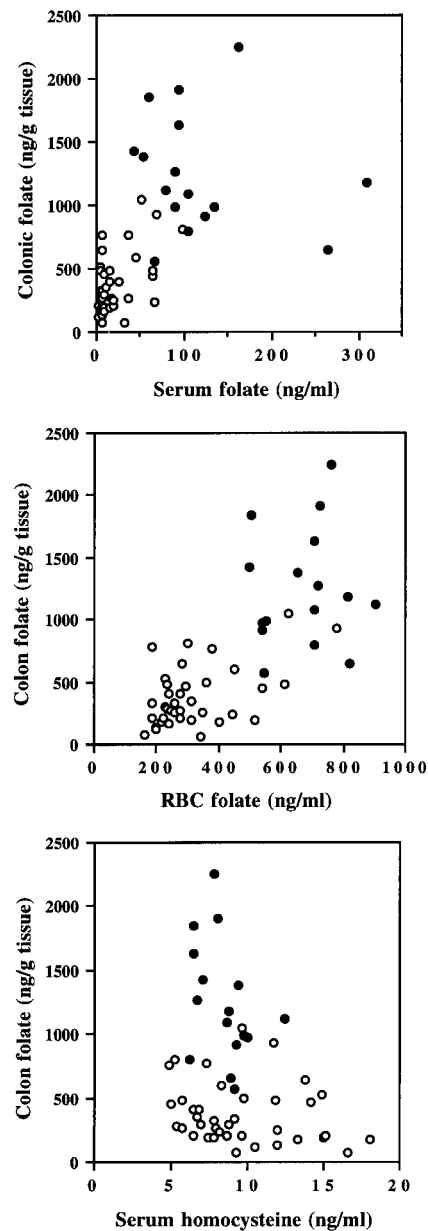


Fig. 1. Serum folate (A), RBC folate (B), and serum homocysteine (C) concentrations from all three time points plotted against colonic mucosal folate concentrations ($n = 55$); ○, 38 samples from subjects not receiving folate; ●, 17 samples from subjects receiving folate supplementation.

(Table 2). Although serum homocysteine concentrations in physiological ranges correlated inversely with colonic mucosal concentrations of folate at baseline ($r = -0.622$; $P = 0.003$), this inverse relationship was no longer observed at 6 months and 1 year (Table 2). Supraphysiological levels of serum homocysteine did not correlate inversely with colonic mucosal folate concentration at each time point (Table 2).

Discussion

The observations from this study indicate that colonic mucosal folate concentrations measured in endoscopic biopsy samples correlate directly with serum and RBC folate concentrations

Table 2 Correlations between colonic mucosal concentrations of folate and serum and RBC folate and serum homocysteine concentrations analyzed separately for subjects receiving folate supplementation and for subjects not receiving folate

| Time | Colonic mucosal concentrations of folate | | | | | |
|--------------------------|------------------------------------------|--------|-------|-----------------|--------|-------|
| | No Folate | | | Folate | | |
| | N | r | P | N | r | P |
| Baseline | | | | | | |
| Serum folate | 20 | 0.662 | 0.001 | NA ^a | NA | NA |
| RBC folate | 20 | 0.385 | 0.309 | NA | NA | NA |
| Serum hcyst ^b | 20 | -0.622 | 0.003 | NA | NA | NA |
| 6 mo | | | | | | |
| Serum folate | 9 | 0.793 | 0.011 | 9 | 0.251 | 0.935 |
| RBC folate | 9 | 0.666 | 0.050 | 9 | 0.031 | 0.515 |
| Serum hcyst | 9 | -0.355 | 0.349 | 9 | -0.149 | 0.702 |
| 1 yr | | | | | | |
| Serum folate | 9 | 0.789 | 0.011 | 8 | 0.492 | 0.262 |
| RBC folate | 9 | 0.683 | 0.042 | 8 | 0.119 | 0.800 |
| Serum hcyst | 9 | -0.316 | 0.408 | 8 | -0.197 | 0.671 |

^a NA, not applicable.

^b hcyst, homocysteine.

and inversely with the concentration of serum homocysteine. These observations corroborate previous animal (6, 7) and human (5) studies, which reported similar findings. However, these data do contrast with the results of two recent human studies in which no correlations between folate concentrations in colonic epithelial cells and serum and RBC folate measurements were observed (8, 9). In the latter studies, viable colonocytes were isolated from biopsies and allowed to equilibrate and continue their cellular metabolism for 4–5 hours before folate concentrations were determined (8, 9). In contrast, our method involves folate determination of a biopsy that has been “snap-frozen” in liquid nitrogen immediately upon removal from the human subject; folate concentrations with our method therefore reflect the folate content at the time of removal from the colon and include folate from the entire milieu of the colonocytes, including lamina propria cells and mucosal mucous. Other than the source of folate [*i.e.*, colonocytes (8, 9) versus colonic mucosa (5)] and tissue folate extraction between these studies, there are no significant differences in methodology and study population which might have contributed to the disparity in results. Future studies will need to be performed to determine which of these methods best reflects folate concentrations of colonocytes *in vivo*, because measurement of folate in the tissue of interest is of considerable importance in the field of folate and cancer (21).

A novel finding from this present study is that, although serum and RBC folate concentrations in conventional physiological ranges correlate well with colonic mucosal folate concentrations, these measurements in supraphysiological ranges resulting from folate supplementation (12.5 times higher than the required daily allowance) did not correlate significantly with colonic mucosal folate concentrations. This observation is probably related to the fact that folate accumulation in tissues is limited by the level of FPGS³ activity in the setting of substrate excess (22, 23). FPGS catalyzes polyglutamation of cellular folates, thereby allowing the retention of folate that would otherwise be lost because of efflux from the cell (22, 23). Previous studies in animals and in cultured cells have shown that tissue levels of folate reach a plateau when FPGS is saturated from excess folate in the diet or culture medium (6,

22, 24). Although the tissue “saturating” blood levels of folate have not yet been determined in humans, a previous animal study has shown that the saturation levels of colonic mucosal folate were reached when serum folate concentrations exceeded ~90 ng/ml (6). A visual inspection of Fig. 1 suggests that serum values exceeding ~100 ng/ml or RBC values exceeding ~800 ng/ml are unlikely to be accurate reflections of colonic folate concentrations. These thresholds are only exceeded when individuals are consuming supraphysiological doses of folate. Taken together, these data therefore suggest that serum and RBC concentrations of folate in supraphysiological ranges may not accurately predict the actual colonic mucosal folate concentrations.

Although serum homocysteine concentrations were the best indicator of colonic mucosal folate concentrations in a previous human study (5), the association between serum homocysteine levels and colonic mucosal folate concentration was weaker than those among serum, RBC, and colonic mucosal folate concentrations in the present study. A significant inverse association between serum homocysteine and colonic mucosal folate concentrations was observed at baseline and when values from subjects on placebo and those receiving folate supplementation were combined at 6 months and 1 year. However, no significant inverse association between these two parameters was observed at 6 months and 1 year when subjects not receiving folate and subjects receiving folate supplementation were separately analyzed. In the parent study (10) from which the present study was derived, serum homocysteine concentrations significantly increased at 6 months and 1 year, for unknown reasons, compared with the baseline values in both the placebo and folate-supplemented groups. This was an unusual finding, given that folate supplementation significantly increased serum (by 2.7–4.4 times), RBC (by 1.9–2.0 times), and colonic mucosal (by 2.2–3.6 times) folate concentrations from the baseline values at 6 months and 1 year, whereas serum, RBC and colonic mucosal folate concentrations did not significantly change over time in the placebo group (10). Therefore, it is possible that factors other than folate might have contributed to this abnormal response in serum homocysteine levels during the study period, which in turn might have affected correlations between serum homocysteine and colonic mucosal folate concentrations. It is well known that a strong, nonlinear, inverse association exists between plasma homocysteine and folate concentrations;

³ The abbreviation used is: FPGS, folypolyglutamate synthetase.

in the upper ranges of plasma folate concentrations, however, plasma homocysteine levels are less affected by plasma folate concentrations (25, 26). This may be the case for the inverse association between serum homocysteine levels and colonic mucosal folate concentrations. Future studies are warranted to better define the relationship between colonic mucosal folate and serum homocysteine levels in both physiological and supraphysiological ranges of serum and RBC folate concentrations.

One potential limitation of the present study is the relatively small number of subjects ($n = 20$ at baseline). This number was reduced further when those not receiving folate and those receiving folate supplementation were separately analyzed at the two follow-up time points. Despite the small sample size, however, we found significant direct correlations between blood measurements of folate and colonic mucosal folate concentrations, and inverse correlations between serum homocysteine and colonic mucosal folate concentrations. The small sample size probably accounts for the lack of consistent correlations between RBC and colonic mucosal folate concentrations (at baseline) and between serum homocysteine and colonic mucosal folate concentrations (when those not receiving folate and those receiving folate supplementation were separately analyzed at 6 months and at 1 year).

At present it is uncertain what constitutes a normal range of folate concentrations in the colonic mucosa, and what is the threshold of colonic mucosal folate concentrations below which colorectal cancer risk increases. These issues need to be resolved, because accurate measures of folate status in the colorectal mucosa are important for identifying target groups of individuals at increased risk of developing colorectal cancer for aggressive screening and for potential folate chemoprevention. Our present data, in conjunction with previous studies (5–7), suggest that serum and RBC folate concentrations in conventional physiological ranges are good indicators of colonic mucosal concentrations. These blood measurements of folate may be used (with some misclassification error) to classify patients into those with low, normal, and high colonic mucosal levels of folate. However, it appears that, once supplementation is initiated, the ability of serum and RBC folate concentrations in supraphysiological ranges to accurately predict colonic mucosal folate concentrations is less reliable.

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