Folate deficiency alters hepatic and colon MGMT and OGG-1 DNA repair protein expression in rats but has no impact on genome-wide DNA methylation

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Abstract

Folate deficiency is implicated in human colon cancer. The effects of feeding rats a folate deficient diet for 24 weeks on DNA damage (8-oxo-7,8-dihydroguanine), DNA repair (MGMT and OGG-1 activity) and epigenetic parameters (genome-wide cytosine methylation and indices of cellular methylation status) were investigated. Relative to control diet, the folate-deficient diet resulted in significantly reduced levels of serum (approx 80%; P<0.0001), whole blood (approx 40%; P<0.0001) and tissue folate levels (between 25-60% depending on the tissue sampled; P<0.05), increased plasma total homocysteine (approx. 35%; P<0.05) and decreased S-adenosylmethionine to S-adenosylhomocysteine concentrations (SAM:SAH, approx. 11%; P<0.05). There was no significant change in the levels of 5-methyldeoxycytidine in liver or colon DNA nor in the activity of liver DNA cytosine methyltransferase. However there was a significant increases in 8-oxo-7,8-dihydroguanine (P<0.001) in lymphocyte DNA and in levels of the DNA repair proteins OGG1 (approx. 27%; P<0.03) and MGMT (approx. 25%;P<0.003) in liver, but not in colon. This may reflect the ability of the liver, but not the colon, to upregulate DNA repair enzymes in response either to elevated DNA damage or an imbalance in the nucleotide precursor pool. These results demonstrate that folate deficiency can significantly modulate DNA damage and DNA repair providing mechanisms by which it plays a role in the aetiology of human cancer. We speculate that the inability of colon tissue to respond to folate deficiency occurs in humans and may increase the potential for malignant transformation.

Keywords

folic acid deficiency in rat; DNA repair; MGMT; 8-oxoguanine-DNA glycosylase; DNA methylation

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Introduction

Folates have a critical role in maintaining DNA stability by donating one-carbon (1-C) moieties. High folate status is associated with a decreased risk of certain malignancies including colorectal cancer (CRC; 1,2). However, an entirely protective role for folate against carcinogenesis has been questioned. Rodent studies report a reduction in early markers of colon cancer when folic acid is given prior to initiation of lesions (2-4). After initiation, folic acid increases carcinogenesis (5). Disturbingly, recent data indicate that an excessive intake of folic acid (from high dose supplements or fortified foods) may increase human cancers, including colon, prostate and breast, by accelerating growth of precancerous lesions, but the dose and timing of such intervention is critical (6-9). However, on balance, evidence from the majority of human studies (retrospective, case-control and prospective) indicate that people who habitually consume the highest level of folate or who have the highest circulating folate have a 40-60% reduced relative risk (RR) of developing polyps or overt CRC (1,2). A recent meta-analysis of prospective studies (10) reported a reduced risk for CRC in subjects with a high dietary folate intake compared with low intake [RR 0.75; 95% CI=0.64-0.89]. Existing evidence, therefore, indicates that increasing folate status in humans is geno-protective except perhaps in individuals with pre-existing disease or who consume supra-nutritional levels of synthetic folic acid (6-9). Suboptimal folate status in humans remains widespread (11,12).

How low folate increases cancer risk remains to be established, although multiple mechanisms have been postulated. Folate deficiency may induce epigenetic effects. Insufficient 5-methyltetrahydrofolate (5-methylTHF), by attenuating remethylation of s-adenosylhomocysteine (SAH) to s-adenosylmethionine (SAM) in the methionine cycle, leads to cytosine demethylation and global DNA hypomethylation. Genome-wide hypomethylation may induce protooncogene activation and chromosomal instability, common features in human tumours (13, 14). Inadequate dietary folate results in decreased intracellular 5, 10-methylenetetrahydrofolate (5, 10-methyleneTHF) which retards conversion of dUMP to dTMP leading to cellular thymidine depletion, uracil misincorporation into DNA, chromosomal breakage and malignant transformation (15, 16). In addition to its effects on thymidine metabolism, folate deficiency impacts on purine biosynthesis by inhibiting 10-formyltetrahydrofolate (10-formylTHF)-mediated production of adenosine and guanosine.

We speculate that folate insufficiency, in addition to inducing DNA hypomethylation and DNA damage, may accelerate genomic instability by impairing DNA repair. There is no question that cancer development is linked to compromised DNA repair. Classic examples of this are the defects in mismatch repair (MMR) genes that are associated with hereditary nonpolyposis colon cancer (HNPCC; 17) and mutations in nucleotide excision repair (NER) genes that underlie syndromes such as *Xeroderma Pigmentosum* with an associated increased risk of skin cancer (18). Moreover, in the general population, specific polymorphisms in DNA repair genes are associated with chromosomal abnormalities (19) and cancer risk (20).

In order to explore the effects of folate status on DNA repair capacity in vivo, we have determined in rats the differential impact of a prolonged folate deficiency on both the hepatic and colon activity of two DNA repair enzymes implicated in the development of human cancers (21-23). We have determined how folate deficiency affects concentrations of 8-oxo-7,8-dihydroguanine and the enzyme that repairs this lesion, 8-oxoguanine-DNA glycosylase (OGG1). The potential for repair of alkylation damage has been assessed via quantitation of O^6-methylguanine-DNA methyltransferase (MGMT). In parallel, to

comprehensively assess the effect of folate deficiency on genomic stability we have quantified global DNA methylation in these tissues.

Materials and Methods

Animals and diets

Amino acid-defined diets devoid of folic acid are an established means of predictably inducing folate deficiency (24, 215). Isoenergetic diets were formulated as described in Table 1. Mineral and vitamin mixes were in accordance with NRC recommendations (25) but were free of folic acid and the antibiotic succinyl sulfathiazole (Table 1). Succinyl sulfathiazole, which inhibits bacterial folate metabolism in the gut, is used in certain studies to induce severe folate deficiency (26, 27). Omission of antibiotic from the diet results in a mild to moderate folate deficiency, allowing the study to be carried out over a longer time period (24).

Male Hooded-Lister (Rowett strain) rats (n=56) were used. These animals are not genetically predisposed towards colon cancer, nor do they show signs of malignant transformation in response to long-term folate deficiency (25). They were weaned at 19 days, group housed and given free access to control diet until they reached 95-100 g [40 days old]. They were then individually housed on grid floors (to prevent coprophagy) and offered a fixed amount (12 g/d) of the same diet for 5 days.

At the beginning of the intervention (week 0), 8 untreated rats (now aged between 6 and 7 weeks) were killed by anaesthetic overdose (Halothane) and exsanguination via cardiac puncture. Blood was taken for plasma, erythrocyte and lymphocyte preparation and tissues for folate analysis.

The remaining rats (n=48) were fed experimental diet [folate sufficient (F+) or folate-free (F−); 24 animals per diet) for up to 24 weeks. We have shown previously that it is possible to detect a highly significant decrease in blood and tissue folate status using these numbers of animals in each group (25).

The rats were given a fixed amount of diet daily throughout the study; initially 12g/rat/d, increasing to 15 g/rat/d after 1 week and to 16.5g/rat/day from 6 weeks onwards in accordance with the growth requirements of the animals. All food was eaten (data not shown). The amount offered was equivalent to 90-100% of normal free intake of semi-synthetic diet. Water was available at all times and the rats were weighed three times weekly.

All procedures were carried out in accordance with the requirements of UK Animals (Scientific Procedures) Act 1986.

Blood folate measured longitudinally throughout the intervention

The effect of intervention on blood folate status was measured longitudinally throughout the study. Samples were collected at week 0, week 8, week 16 and week 24 post intervention. Whole blood (approx. 0.5ml) was collected from the tail vein, "snap frozen" in liquid nitrogen and stored at −80°C or centrifuged at $2400 \times g$ for 15 min at 4°C. Plasma was aliquoted, "snap frozen" and stored. Plasma and whole blood folate were determined in these samples by radioassay (25).

Blood and tissue collection post-intervention

After 24 weeks the rats were killed by anaesthetic overdose and exsanguination via cardiac puncture, and blood and tissues (brain, colon, kidney, liver and spleen) collected. Whole

blood from all animals was sampled by cardiac puncture and either "snap frozen" in liquid nitrogen and stored at −80°C for analysis or centrifuged at 2400 × g for 15 min at 4°C. Plasma was aliquoted, "snap frozen" and stored. Lymphocytes from whole blood were prepared for folate analysis or quantification of DNA damage (described below). Cell number (lymphocytes) was determined using a haemocytometer prior to freezing. Brain, kidney and spleen were "snap frozen" whole in liquid nitrogen for folate analysis. Colon from half the animals in each group (n=12) was perfused with ice-cold Tris-sucrose, opened flat and dissected into proximal, transverse and distal sections for folate, B12, DNA methylation and SAM:SAH analysis. Liver from the same animals was perfused with ice-cold Tris-sucrose, blotted and divided into individual lobes for analysis of the same biomarkers and dnmt activity. Colon and liver from the remaining animals (n=12) was collected for DNA repair activity. All samples were "snap frozen" as above.

Oxidative DNA damage and misincorporated uracil

DNA damage was measured in rat lymphocytes at the end of the experiment. Whole blood (30μl) was resuspended in RPMI 1640 medium supplemented with 10% (v/v) FCS, underlain with LymphoPrep and centrifuged at 200 × g for 4.5 min at 4°C. The lymphocyte-rich buffy coat (>90% lymphocytes) was removed and analysed immediately for DNA damage. Misincorporated uracil and 8-oxo-7,8-dihydroguanine were measured specifically using alkaline single cell gel electrophoresis (SCGE) combined either with formamidopyrimidine glycosylase (FPG), the bacterial equivalent of mammalian OGG1 protein (21) or uracil DNA glycosylase incubation as described previously (25).

Oxidative and alkylation DNA repair protein activity (OGG-1 and MGMT)

Repair activities of 8-oxoguanine-DNA glycosylase (OGG1), which catalyses the removal of mutagenic 8-oxo-7,8-dihydroguanine from DNA, and O^6-alkylguanine-DNA alkyltransferase (methylguanine methyltransferase; MGMT), which repairs toxic, mutagenic and carcinogenic O^6-guanine damage, were determined in liver and colon scrapings by [^{32}P]-labelled oligonucleotides cleavage assays. MGMT activity was determined by incubation of tissue sonicate (28) with a [^{32}P]-labelled oligonucleotide containing O^6-meG within a PstI recognition site attached via 3′biotin to the wells of streptavidin coated plates. Repair of the O^6-meG by MGMT results in deprotection of the restriction site and, upon incubation with PstI, release of a short radio-labelled oligonucleotide fragment into the supernatant. MGMT activity is proportional to the amount of radioactivity present in the supernatant, quantified in a TOPCOUNT machine (Perkin Elmer). OGG1 activity was quantified in a similar way by measuring direct cleavage of an immobilised 8-oxoguanine containing-oligonucleotide (29).

Methyl group status, DNA methyltransferase activity and genomic DNA methylation

Blood samples were collected after 24 weeks on the folate-sufficient or folate-deficient diets and plasma tHcy measured by reverse phase HPLC using a DS30 Hcy Homocysteine Assay Kit in combination with a DS30 analyser (Drew Scientific, Barrow-in-Furness, UK). Liver and distal colon SAM and SAH were measured in PCA-treated samples by HPLC (30). Blood and tissue B12 levels were measured by radioassay (25). Liver Nuclear proteins were prepared using a kit (EpiQuik Nuclear Extraction Kit1; Epigentek, NY, USA). DNA methyltransferase (Dnmt) activity in these extracts was measured colorimetrically (EpiQuik DNA Methyltransferase Activity/Inhibition Assay Kit; Epigentek, NY, USA). DNA was isolated from rat liver and colon using a Nucleospin C&T kit (Abgene Ltd, Epsom, UK). 5-methyl cytosine levels were was quantified by LC MS/MS (31).

Statistical Analysis

Data are presented as mean +/− SEM with number of animals in parenthesis. Significant differences between treatment groups were analysed by Students' t-test using SPSS (version 13).

Results

Folate status

Rats were fed either a control diet containing folic acid (5mg/kg diet) or a diet devoid of folic acid for up to 24 weeks. Folate deficiency had no significant effect on body weight (Figure 1). No behavioural or pathological abnormalities were observed in folate-deficient animals compared with controls.

In animals fed the folate-free diet, plasma folate significantly decreased to an 8 week low, which was maintained for the duration of the study, resulting in approx. 80% of control levels by 24 weeks. There was an approx. 40% decrease in whole blood folate by 24 weeks (Figure 2). Lymphocyte folate decreased 40% and uracil misincorporation increased 2-fold over the same period (Table 2, Figure 3).

Tissue folate declined between 25% and 60% (depending on the organ) in animals fed the folate-deficient diet. Folate depletion was similar for all three colon regions (approx. 60%). Brain (25% of controls) and liver (31% of controls) were the organs least affected by dietary folate deficiency (Table 2).

Oxidative DNA damage

Intracellular folate depletion in rat lymphocytes was associated with a significant increase in levels of the oxidised purine base, 8-oxo-7,8-dihydroguanine (Figure 3).

DNA repair protein activity

Folate deficiency increased MGMT (25%) and OGG-1 (27%) activity in rat liver (P=0.03 and 0.003 respectively; Figure 4A). In the distal colon, these DNA repair activities were unaffected by folate status (Figure 4B) although there was a trend for decreased OGG-1 activity (P=0.054) in folate-deficient tissue.

Methyl group status, DNA methyltransferase activity and genomic DNA methylation

Folate deficiency increased plasma homocysteine (approx. 25%, P<0.02; Table 3). There was no effect of folate deficiency on B vitamin status (plasma, lymphocyte, liver or colon). The ratio of hepatic SAM to SAH was significantly decreased in rats fed a folate-free diet for 24 weeks (approx. 11%; P<0.004; Table 3). Folate deficiency caused a similar drop in SAM:SAH in the distal colon (Table 2). Dnmt activity was elevated in folate-deficient rat liver (Table 3). DNA was hypomethylated in the liver and colon from folate deficient animals compared with controls (Table 3). 5-methyldeoxycytidine concentrations and percentage methylated DNA (measured by LC MS/MS) were both lower in these tissues. However, none of these differences reached statistical significance.

Discussion

Folate is essential for the biosynthesis, repair and methylation of DNA. High folate status (based on measures of dietary intake and blood levels) is generally associated with a decreased risk of certain human cancers, including colorectal cancer (1, 2). However, there is real concern regarding a potential harmful role for long-term intervention with high doses of synthetic folic acid. Findings from recent human observational and placebo-controlled

intervention trials, or analyses of cancer incidence data, suggest that supplementation with synthetic folic acid may promote progression of initiated cancer cells at several sites including the breast, colon and prostate (6-9).

Nonetheless, evidence from the majority of studies indicates that habitual consumption of natural folates from the diet reduces cancer development, while folate deficiency increases risk (1, 2, 10). Folate deficiency perturbs nucleotide synthesis, increases DNA damage and alters global and gene-specific methylation. The impact that folate deficiency has on DNA repair in vivo has not been extensively examined.

Defective DNA repair is linked to human cancer development (17, 18). Mutations in MMR genes are associated with heritable colon cancer (17) while polymorphisms in specific repair genes (e.g. hOGG1, XRCC1 and PARP1) correlate with altered cancer risk and progression of colorectal cancer (20, 32). Folate is essential for the repair of DNA. In the present study we investigated whether a moderate folate deficiency in rats altered the repair capacity for two lesions implicated in human cancer aetiology. The strongly mutagenic oxidised base, 8-oxo-7,8-dihydroguanine occurs in significant quantities in human DNA and induces G:C to T:A transitions (21). Removal of this lesion by BER is achieved by 8-oxoguanine-DNA glycosylase (OGG1). O^6-methylguanine is detectable in human colonic DNA (22) and at high levels in tumour prone regions of the human bowel (23). It is potentially cytotoxic, mutagenic (causing G:C to A:T transitions and recombinations) and carcinogenic unless repaired by MGMT. Decreased activity of MGMT in normal human colorectal tissue and cancerous tissue is strongly associated with G:C to A:T mutations in the K-ras protooncogene (23).

We firstly showed that feeding rats a folate-deficient diet for 24 weeks caused moderate intracellular folate deficiency. Blood cell folate dropped by 40% and tissue folate by 25-60%. Liver folate was depleted approx. 30% and colon 60%. These data are consistent with a study carried out by us in rats fed the same diet for 8 weeks where whole blood and liver folate decreased 29% and 26% respectively (25). Similar folate levels have been reported in rats fed a folate-free diet (without succinyl sulfathiazole) for 15 weeks. Here, serum folate was reduced by 54%, liver folate by 67% and colon folate by 30%. This was described as a mild folate deficiency (24). Conversely, feeding rodents succinyl sulfathiazole, together with a folate-free diet, induces a much more severe deficiency. Plasma folate was depleted 93% over 8 weeks in DNA repair deficient mice fed this diet (26) and liver folate by 95% in normal mice after only 4 weeks (27). In addition to the effects of the deficient diet on folate levels we also observed a 2-fold increase in uracil misincorporation. In this study, uracil misincorporation served as a sensitive intracellular marker of folate status and function. We have shown previously that uracil misincorporation in vitro and in rats is increased in a concentration or time-dependent manner (25, 33, 34). Uracil is also present at detectable levels in human DNA (31) and is increased by folate depletion (15) and reduced by supplementation (35).

We have reported recently that DNA repair enzymes are significantly upregulated in folate-depleted cells in vitro (33). Proteomic analysis of human NCM460 cells grown in folate-deficient medium showed significant changes in expression of DNA repair enzymes including MSH2 and XRCC5, involved in MMR and double strand break (DSB) repair respectively. Despite this upregulation in repair enzyme activity, BER of oxidation and alkylation damage is compromised in folate-deficient human lymphocytes, in normal human colon epithelial cells and in rat colon cells ex vivo (24, 33, 34). Increased repair enzyme expression appears counterintuitive given the evidence that folate deficiency profoundly inhibits repair in vitro (24, 33, 34). However, single cell gel electrophoresis (SCGE), the assay used in these in vitro experiments, assesses repair completed by the full complement

of enzymes contained within the cell and not only the initial excision steps in the repair process. It may be that while folate deficiency upregulates the activity of initial DNA incision repair enzymes, other enzymes, for example, DNA polymerases downstream in the repair process remain unchanged and thus complete repair is compromised. Damaged bases are removed by DNA glycosylases in BER and upregulation of certain of these repair enzymes has been reported in repair-deficient mice, without a corresponding increase in β-polymerase activity (26). Increased incision and excision without subsequent patch repair would increase DNA strand breakage. This, together with depletion of the nucleotide precursor pool and/or changes in the balance of DNA precursors as a consequence of folate deficiency (36, 37), would result in the accumulation of DNA strand breaks as observed by Cabelof et al., (26) and detected as DNA repair inhibition in the SCGE assay (24, 33, 34).

An important and novel finding of this study was that folate deficiency significantly increased hepatic OGG-1 and MGMT repair activity (approx. 25% for both proteins). Exactly how folate deficiency modulates DNA repair remains to be established. BER (26) and particularly rat MGMT are DNA damage-inducible (38) and a wide range of genotoxic agents are able to elicit this response most extensively in liver (39). From such observations, it is reasonable to suggest that upregulation of these two proteins indicates the occurrence of DNA damage lending further support to the finding that increased DNA damage (including DNA strand breaks, uracil misincorporation and oxidised bases) is a consequence of folate deficiency (15, 25, 34). Although we attempted to quantify the levels of O^6-methylguanine and 8-oxo-7,8-dihydroguanine directly in rat liver and colon DNA, they were below the limit of detection for the assays employed (data not shown). We cannot therefore report whether MGMT and OGG-1 activity were elevated by increased concentrations of substrate lesions specifically in these tissues. However, folate deficiency significantly increased 8-oxo-7,8-dihydroguanine levels in DNA in lymphocytes from rats fed the folate deficient diet.

Although there were highly significant changes in OGG-1 and MGMT expression in rat liver in response to folate depletion, no such effects were seen in colon, indicating that the ability of the liver to respond to folate deficiency is not shared by the colon. Given the evidence that hepatic upregulation is triggered by DNA damage, it is reasonable to speculate that the colon cannot respond as robustly as the liver to such damage and would therefore be more susceptible to the genotoxic effects instigated by folate deficiency. An alternative explanation, that colon does not suffer the same extent of DNA damage as the liver, seems less likely.

A key question is whether the findings from this animal study can be extrapolated to humans. Little is known currently about the effect of folate status on DNA repair in humans. NER is impaired in lymphocytes from individuals with poor folate status (40). Conversely, supplementing healthy volunteers of adequate folate status with folic acid (1.2mg/day for 12 weeks) does not alter BER-mediated excision of 8-oxo-7,8-dihydroguanine from lymphocytes (35). While these data from surrogate tissues suggest that DNA repair activity is suboptimal in people with low folate intake andmay not be improved in individuals with satisfactory folate status, a decrease in BER post-intervention was observed in subjects with the lowest baseline red cell folate (35), suggesting that DNA repair may be sensitive to both positive and negative changes in folate status. Whether the colon is less refractive to folate deficiency in people and whether this has a negative impact on genomic stability and malignant transformation remains to be discovered. We have shown previously that MGMT activity in normal human colorectal mucosa is inversely associated with vegetable consumption and that high dietary folate intake is related to low DNA alkylation damage (N7-methylguanine; 41). Similarly, markers of microsatellite instability (MSI) are decreased

in patients with ulcerative colitis (a condition predisposing to CRC) treated with very high doses of folic acid (5mg/day for 6 months; 13).

To complement the repair aspect of this study, we determined the impact of folate deficiency on key intermediates in the methionine cycle (tissue SAM and SAH, plasma homocysteine, plasma, lymphocyte and tissue B12) and genome-wide DNA methylation, a common feature in tumorigenesis (reviewed in 2, 14). Liver and colon DNA was hypomethylated approx. 2% and 5% respectively in rats fed a folate deficient diet relative to DNA from animals in the control group. However, despite a progressive reduction in blood and tissue folate concentrations, an increase in plasma homocysteine and a decrease in the liver SAM to SAH ratio, folate deficiency did not statistically significantly alter genome-wide DNA methylation in these tissues.

The reported effect of folate deficiency on DNA methylation is highly variable and profoundly dependent upon the treatment regime, tissue and genes examined (reviewed in 2). Generally, severe folate deficiency in vitro, in rodents and in humans causes DNA hypomethylation, while moderate deficiency is ineffective (reviewed in 2, 33, 42-44). Our data do not support the hypothesis that folate deficiency induces measurable DNA hypomethylation in vivo suggesting that epigenetic changes are not relevant to any biological effect of folate modulation in our rat model.

In conclusion, moderate but prolonged folate deficiency significantly altered DNA repair activity in rat liver but not in colon. This may reflect the ability of the liver, but not the colon, to upregulate DNA repair enzymes in response to elevated DNA damage. If this inability of colon tissue to upregulate DNA repair processes occurs in humans, it may constitute one of the mechanism through which folate deficiency increases the potential for malignant transformation.

Acknowledgments

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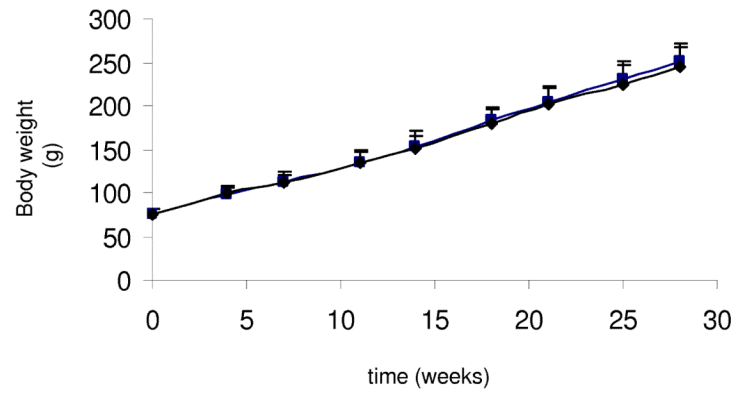
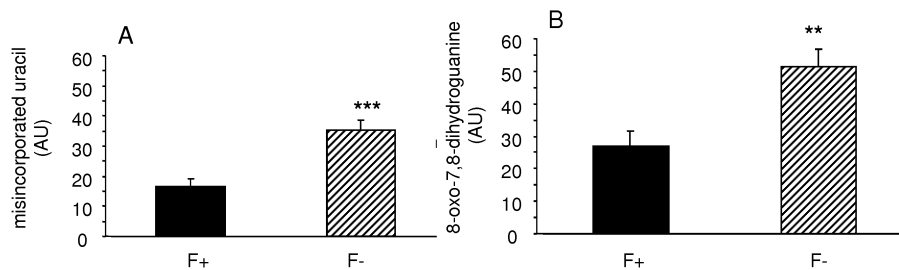**Figure 1. The effect of folic acid deficiency on rat growth**
Rats were either fed a folate sufficient diet (F+; diamonds) or a diet deficient in folic acid (F
−; squares) for up to 24 weeks. Results are mean body weight +/− SEM (n=24 per group).
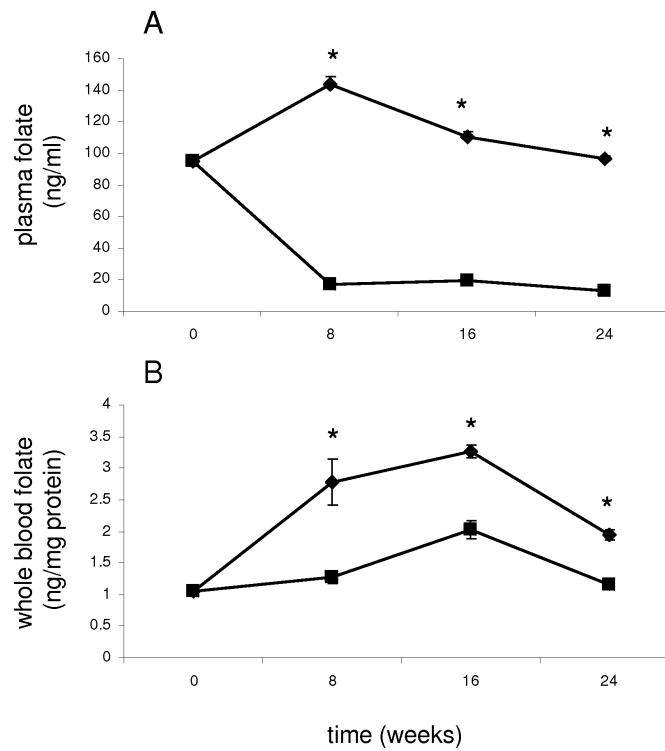
Figure 2. The longitudinal effect of folic acid deficiency on (A) plasma and (B) whole blood folate levels in rats

Rats were either fed a folate sufficient diet (F+; diamonds) or a diet deficient in folic acid (F−; squares) for up to 24 weeks. Results are mean +/− SEM (n=10 per group).
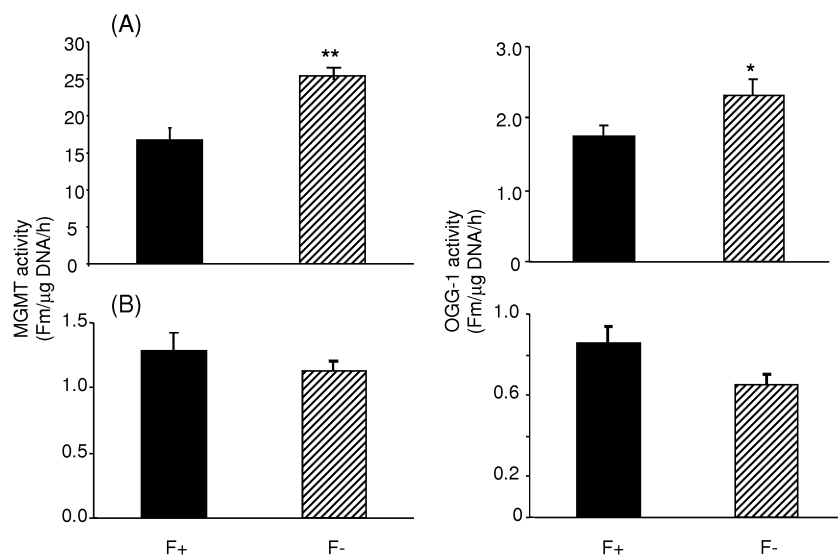
*P<0.0001, where significance refers to differences between F+ and F− treated rats at each time point.

Figure 3. The effect of folic acid deficiency on (A) misincorporated uracil and (B) 8-oxo-7,8-dihydroguanine levels in rat lymphocytes

Rats were either fed a folate sufficient diet (F+; solid bar) or a diet deficient in folic acid (F−; hatched bar) for 24 weeks. Results are mean values (in arbitrary units) +/− SEM (n=8 per group).

**P<0.003; ***P<0.001, where significance refers to differences between F+ and F− treated rats at each time point.

Figure 4. The effect of folic acid deficiency on MGMT and OGG-1 DNA repair activity in (A) rat liver and (B) colon
Rats were either fed a folate sufficient diet (F+; solid bar) or a diet deficient in folic acid (F−; hatched bar) for 24 weeks. Results are mean +/− SEM (n=12 per group).
*P<0.03; **P<0.003, where significance refers to differences between F+ and F− treated rats at each time point.

Table 1

Composition of Experimental Diets (g/kg)

Diet	F+	F−
	(folate-sufficient)	(folate-deficient)
Casein	112	112
Maize starch	382	382
Potato starch	100	100
Glucose	150	150
Corn oil	150	150
Minerals [*]	50	50
Vitamins [*]	50	50
Sodium silicate	0.2	0.2
L-methionine	**4**	**4**
Folic acid mix	**0.005**	**0**
Choline chloride	**2**	**2**

[*] mineral and vitamin mixes were prepared as described (ref 21) with the exception that folic acid was absent.

Table 2

Folate concentrations in lymphocytes and tissues from rats fed a folate supplemented (F+) or folate deficient (F−) diet for 24 weeks.

	folate (ng/mg protein)		depletion (%)
	F+	**F−**	
lymphocyte (ng/10^5 cells)	0.45 +/− 0.08 (8)	0.27 +/− 0.03 [*] (8)	39
liver	136.2 +/− 6.9 (10)	93.9 +/− 16.3 [*] (10)	31
colon (ascending)	25.7 +/− 4.2 (10)	11.9 +/− 1.1 [**] (10)	54
colon (transverse)	27.4 +/− 3.2 (9)	10.5 +/− 1.6 [***] (10)	62
colon (descending)	24.6 +/− 1.9 (10)	9.9 +/− 1.8 [****] (10)	60
spleen	16.5 +/− 2.9 (10)	7.9 +/− 1.1 [**] (10)	52
kidney	58.6 +/− 7.5 (10)	26.6 +/− 2.7 [***] (10)	55
brain	20.4 +/− 0.5 (6)	15.3 +/− 2.2 [*] (6)	25
heart	9.2 +/− 1.2 (10)	3.4 +/− 0.3 [***] (10)	63

Values are means +/− SEM with number of rats sampled per group in parentheses. Significance refers to differences between F+ versus F− animals at the end of the experiment

[*] $P < 0.05$

[**] $P < 0.01$

[***] $P < 0.001$

[****] $P < 0.0001$

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Table 3

Indices of methyl donor status and global DNA methylation in blood and tissues from rats fed a folate supplemented (F+) or folate deficient (F−) diet for 24 weeks.

	Methylation status	
	F+	**F−**
plasma tHcy (μM)	5.01 +/− 0.27 (12)	6.91 +/− 0.32 [*] (12)
plasma B12 (pg/ml)	503.1 +/− 40.5 (10)	579.3 +/− 53.5 (10)
lymphocyte B12 (pg/10^5 cells)	13.94 +/− 0.40 (10)	12.39 +/− 0.70 (10)
liver B12 (pg/mg protein)	366.8 +/− 27.1 (10)	437.8 +/− 44.7 (10)
distal colon B12 (pg/mg protein)	622.7 +/− 50.5 (10)	618.2 +/− 78.6 (10)
liver SAM	81.60 +/− 4.93 (10)	79.22 +/− 4.99 (10)
Liver SAH	14.53 +/− 0.9 (10)	15.78 +/− 1.06 (10)
liver SAM:SAH ratio	5.63 +/− 0.12 (10)	5.04 +/− 0.13 [**] (10)
colon SAM	32.47 +/− 4.36 (10)	28.36 +/− 2.91 (10)
colon SAH	3.57 +/− 0.47 (10)	4.48 +/− 0.52 (10)
colon SAM:SAH ratio	9.75 +/− 1.34 (10)	7.09 +/− 1.03 (10)
liver dnmt (OD/h/mg nuclear protein	4.38 +/− 0.90 (12)	6.70 +/− 2.33 (12)
liver 5-methyldeoxycytidine (ng/μg DNA; LC MS/MS)	2.62 +/− 0.32 (12)	2.12 +/− 0.23 (12)
liver genomic DNA methylation (%; LC MS/MS)	4.63 +/− 0.10 (12)	4.54 +/− 0.04 (12)
colon 5-methyldeoxycytidine (ng/μg DNA; LC MS/MS)	4.56 +/− 0.22 (12)	3.47 +/− 0.51 (12)
colon genomic DNA methylation (%; LC MS/MS)	4.87 +/−0.09 (12)	4.61 +/− 0.15 (12)

Values are means +/− SEM with number of rats sampled per group in parentheses. Significance refers to differences between F+ versus F− animals at the end of the experiment

[*] P=0.02

[**] P=0.004.

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