Nutrition and Cancer
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/hnuc20

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Published online: 18 Nov 2009.

To cite this article: Susan J. Duthie , Sabrina Narayanan , Stephanie Blum , Lynn Pirie & Gillian M. Brand (2000) Folate Deficiency In Vitro Induces Uracil Misincorporation and DNA Hypomethylation and Inhibits DNA Excision Repair in Immortalized Normal Human Colon Epithelial Cells, Nutrition and Cancer, 37:2, 245-251, DOI: 10.1207/S15327914NC372_18

To link to this article: http://dx.doi.org/10.1207/S15327914NC372_18

NUTRITION AND CANCER, *37*(2), 245–251

Folate Deficiency *In Vitro* Induces Uracil Misincorporation and DNA Hypomethylation and Inhibits DNA Excision Repair in Immortalized Normal Human Colon Epithelial Cells

Susan J. Duthie, Sabrina Narayanan, Stephanie Blum, Lynn Pirie, and Gillian M. Brand

Abstract: *Epidemiological studies have indicated that folic acid protects against a variety of cancers, particularly cancer of the colorectum. Folate is essential for efficient DNA synthesis and repair. Moreover, folate can affect cellular* S-*adenosylmethionine levels, which regulate DNA methylation and control gene expression. We have investigated the mechanisms through which folate affects DNA stability in immortalized normal human colonocytes (HCEC). DNA strand breakage, uracil misincorporation, and DNA repair, in response to oxidative and alkylation damage, were determined in folate-sufficient and folate-deficient colonocytes by single cell gel electrophoresis. In addition, methyl incorporation into genomic DNA was measured using the bacterial enzyme Sss1 methylase. Cultured human colonocyte DNA contained endogenous strand breaks and uracil. Folate deficiency significantly increased strand breakage and uracil misincorporation in these cells. This negative effect on DNA stability was concentration dependent at levels usually found in human plasma (1–10 ng/ml). DNA methylation was decreased in HCEC grown in the absence of folate. Conversely, hypomethylation was not concentration dependent. Folate deficiency impaired the ability of HCEC to repair oxidative and alkylation damage. These results demonstrate that folic acid modulates DNA repair, DNA strand breakage, and uracil misincorporation in immortalized human colonocytes and that folate deficiency substantially decreases DNA stability in these cells.*

Introduction

Colorectal cancer is the second most common cancer in the population of developed countries (1,2). A diet low in vegetables and fruit is associated with increased colorectal cancer risk (3). Several epidemiological studies have reported an inverse association between dietary folate intake and colorectal cancer incidence (4,5). In a recent study (6), folate intakes >400 μg/day were associated with a significantly reduced risk for colon cancer [relative risk (RR) = 0.69, 95% confidence interval (CI) = 0.52–0.93]. Long-term use of folate supplements was reported to reduce the risk of malignancy even further (RR = 0.25, CI = 0.13–0.51). Serum folate is also inversely related to colorectal cancer incidence (7). In a nested case-control study of 105 colorectal cancer patients and 523 matched controls, the risk of malignancy was 50% lower in individuals grouped in the highest quartile of serum folate than in those in the lowest quartile. Folate supplementation also protects against neoplasia in high-risk individuals with ulcerative colitis (8).

Folate is crucial for normal DNA synthesis and repair. It is also a cofactor in the metabolism of homocysteine to *S*-adenosylmethionine (SAM), the primary intracellular methyl donor (9). Folate deficiency may decrease DNA stability and increase the risk of malignant transformation, either by perturbing the nucleotide pool and negatively altering DNA synthesis and repair or by disrupting DNA methylation, leading to altered gene transcription and protooncogene expression (10,11).

Our understanding of mutagenesis, tumorigenesis, and metastasis in several tissues has been substantially enhanced by culture systems comparing malignant and nonmalignant cells (12–14). Culture models have proved valuable in the investigation of protooncogene expression, growth factor production, proliferation, and angiogenesis (15–17).

Although epidemiological data strongly suggest a link between dietary folate intake and colorectal cancer risk, studies investigating potential mechanisms of DNA instability have been limited because of the lack of suitable *in vitro* cell culture models.

Simian virus-40 (SV40) transfection and immortalization of human and animal cells have provided useful laboratory models for investigating malignant transformation, aging, and metabolism (18–20). Activation and toxicity of xenobiotics are extensively studied in immortalized hepatocytes or bronchial cells, which retain metabolic competence (21,22). Immortalized enterocytes and bile duct epithelial cells have provided data on intestinal drug transport, normal lipid metabolism, and bile acid production

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(20,23). Moreover, SV40-immortalized dopamine-producing rat neurons have successfully been used in transplant therapy against an experimental model of Parkinson disease (24).

We report here the influence of folate status on DNA stability, DNA methylation, and DNA repair in nonmalignantly transformed human colon epithelial cells (HCEC).

Materials and Methods

Materials

Complete A52 medium (4 mg/l folic acid) and A52 medium without folic acid (<1 ng/ml), bovine pituitary extract, retinoic acid, and soybean trypsin inhibitor were supplied by Biofluids (Rockville, MD), Nucleospin C & T kits for DNA isolation from BioGene (Cambridge, UK), 4′,6-diamidine-2-phenylindole dihydrochloride (DAPI) from Boehringer-Mannheim (Lewes, UK), uracil DNA glycosylase from Helena Biosciences (Sunderland, UK), and Matrigel basement membrane matrix from Becton (Bedford, UK). Ultrapure low-melting-point (LMP) and electrophoresis-grade high-melting-point agarose and Nunc sterile tissue culture flasks were obtained from GIBCO Life Technologies (Paisley, UK). Simultrac Radioassay Kit [^{57}Co]vitamin B-12-[^{125}I]folic acid was obtained from ICN Flow (Irvine, UK) and ^{3}H-labeled *S*-adenosyl-L-methionine from NEN Life Sciences (Hounslow, UK). The cytochrome *P*-450 inducers retinoic acid and dexamethasone were obtained from Sigma Chemical (Poole, UK).

Methods

Routine culture of HCEC: Primary adult colon cells were obtained by scratch biopsy from a 69-year-old female donor undergoing surgery for sigmoidal diverticulitis and immortalized using SV40 T antigen (25). These cells retain many intestine-specific characteristics and functions, including expression of cytokeratins, alkaline phosphatase and cytokines, and growth factors specific to normal colon tissue (25). They display microvilli and form electron-dense junctional complexes. Cytochrome *P*-450 expression and phase II enzyme activity are identical to those found in normal HCEC (25).

HCEC were maintained in complete A52 medium supplemented with L-glutamine (2 mM), retinoic acid (100 nM), dexamethasone (1 nM), vitamin C (38 μg/ml), folic acid (4 mg/l), and bovine pituitary extract (30 μg/ml). Cells were incubated at 37°C in an atmosphere of 95% air-5% CO_2. Culture medium was changed every three to four days, and the stocks were passaged into 75-cm^2 flasks precoated with human connective tissue matrix (Matrigel) at a ratio of 1:5.

Culture of HCEC for folate-deficiency studies: Flasks (25 cm^2) were coated with 0.3 ml of cold (4°C) Matrigel by use of chilled pipettes and incubated at 37°C for up to one hour. Excess Matrigel was removed, and the flasks were washed with sterile phosphate-buffered saline (PBS). HCEC were subcultured using trypsin-ethylenediaminetetraacetic acid (EDTA) solution (0.25% trypsin in 0.02% EDTA), and the reaction was stopped using soybean trypsin inhibitor. The cells were washed three times in PBS, plated at a density of 1×10^4 cells/flask, and allowed to grow in folate-deficient or folate-sufficient medium for up to 14 days. In certain experiments, HCEC were incubated in folate-deficient medium or in medium supplemented with 1, 5, or 10 ng/ml folic acid. The effect of folic acid status on oxidative (H_2O_2) and alkylation [methyl methanesulfonate (MMS)] damage and repair was determined in HCEC grown in folate-replete or folate-deficient medium. To determine the toxicity of these compounds, HCEC were exposed to H_2O_2 (0–200 μM) on ice for 5 minutes or MMS (0–5 mM) at 37°C for 30 minutes. To measure repair, the cells were immediately sampled for comet analysis after treatment with H_2O_2 (50 μM) or MMS (5 mM) or incubated in complete culture medium (folate sufficient or folate deficient, as appropriate) at 37°C in 95% air-5% CO_2 for up to 24 hours. In all cases, the cells were isolated from the flasks with use of trypsin-EDTA, centrifuged at 200 *g* for two minutes at 4°C, and resuspended in 80 μl of LMP agarose for comet analysis. Cell growth was determined using a Neubauer Improved Hemocytometer.

Single-cell gel electrophoresis: DNA strand breakage, misincorporated uracil, and DNA repair (in response to H_2O_2 or MMS treatment) were measured using the alkaline comet assay or single-cell gel electrophoresis, as described previously (26). Strand breakage causes relaxation of the supercoiling in the DNA molecule, allowing DNA loops to be pulled toward the anode during electrophoresis. Inclusion of the bacterial DNA repair enzyme DNA glycosylase enables misincorporated uracil to be detected. Fluorescence staining enables DNA damage (the comet tail) to be visualized and measured (26). Isolated HCEC were resuspended in 80 μl of 1% LMP agarose (wt/vol in PBS, pH 7.4) and pipetted onto a frosted glass microscope slide precoated with a similar solution and volume of high-melting-point agarose. The agarose was set for 10 minutes at 4°C, and the slide was incubated for 1 hour at 4°C in lysis solution [2.5 M NaCl, 10 mM tris(hydroxymethyl)aminomethane (Tris), 100 mM Na_2EDTA, and NaOH to pH 10.0] containing 1% (vol/vol) Triton X-100. The slide was washed three times for five minutes each in uracil DNA glycosylase buffer [60 mM Tris·HCl, 1 mM EDTA, 0.1 mg/ml bovine serum albumin, pH 8.0] and gently blotted with tissue paper. The gel was covered with 50 μl of buffer or uracil DNA glycosylase (enzyme), sealed with a glass coverslip, and incubated at 37°C for one hour in a moist atmosphere. The slides were then aligned in a horizontal gel electrophoresis tank (260 mm wide) containing buffer (0.3 M NaOH and 1 mM Na_2EDTA, pH 12.7) for 40 minutes before electrophoresis at 25 V for 30 minutes. The slides were washed three times for five minutes each at 4°C in neutralizing buffer (0.4 M Tris·HCl, pH 7.5) and stained with DAPI.

DAPI-stained nucleoids (20 μl of a 5 μg/ml stock solution) were scored visually (as the intensity of fluorescence in

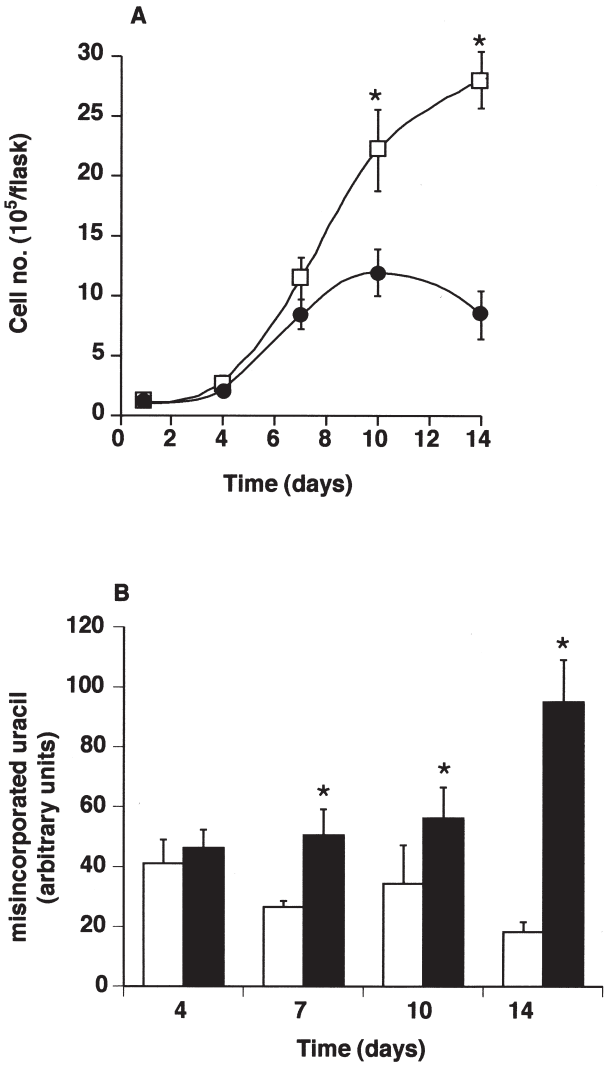

Figure 1. Effect of folic acid deficiency on human colon epithelial cell (HCEC) proliferation (A) and uracil misincorporation (B). HCEC were grown in presence (*open symbols*) or absence (*closed symbols*) of folic acid (4 mg/l) for up to 14 days. Values are means ± SEM; $n > 8$. *, $p < 0.05$ vs. absence of folic acid.

the comet tail). One hundred comets were scored per slide based on the amount of fluorescence in the comet tail and assigned a value of 0, 1, 2, 3, or 4 (from undamaged Class 0 to maximally damaged Class 4). The total score (in arbitrary units) could, therefore, range from 0 (all undamaged) to 400 (all maximally damaged). This method of visual classification has been extensively validated using computerized image analysis (Komet 3.0, Kinetic Imaging, Liverpool, UK), as published previously (26).

Where gels were not exposed to buffer or enzyme (H_2O_2 and MMS toxicity and DNA repair), the slides were taken immediately from lysis to unwinding and electrophoresis before DAPI staining as normal.

HCEC DNA isolation and genomic DNA methylation: HCEC genomic DNA was isolated using the commercially available NucleoSpin C & T kit. DNA concentration was determined spectrophotometrically at 260/280 nm. Genomic DNA methylation was measured in isolated DNA after the method of Balaghi and Wagner (27). This assay measures the incorporation of methyl groups from ^{3}H-labeled *S*-adenosyl-L-methionine at cytosine residues in genomic DNA by use of the bacterial enzyme Sss1 methylase. The extent of DNA methylation is inversely related to the degree of radioactive incorporation, i.e., the lower the methylation status of the DNA the higher the disintegrations per minute.

Statistics: Analysis of variance and Student's *t*-test were carried out as appropriate by use of Statistical Package for Social Sciences (SPSS version 8).

Results

HCEC cultured in folate-deficient medium displayed progressively retarded growth compared with colon cells incubated with folic acid (Figure 1A). Misincorporated uracil levels increased significantly above background levels in HCEC cultured for 7–14 days in folate-deficient medium (Figure 1B). Severe folate deficiency was also associated with a decrease in global DNA methylation (Figure 2).

The effects of folate deficiency on cell proliferation and uracil misincorporation were concentration dependent (Figure 3). However, DNA hypomethylation (as a result of extreme folate deficiency) was similar at all folate concentrations (data not shown).

H_2O_2 and the alkylating agent MMS increased DNA strand breakage above endogenous levels (Figure 4). HCEC cultured for 14 days in folate-deficient medium were unable to repair effectively H_2O_2- or MMS-induced damage after 8 and 24 hours (Figure 5). This was clearly seen when the frequency of comets in the five different classes after treatment with MMS was compared (Figure 6). HCEC, cultured in the presence or absence of folic acid, responded equally to the alkylating agent (0 h). However, folate-deficient colonocytes

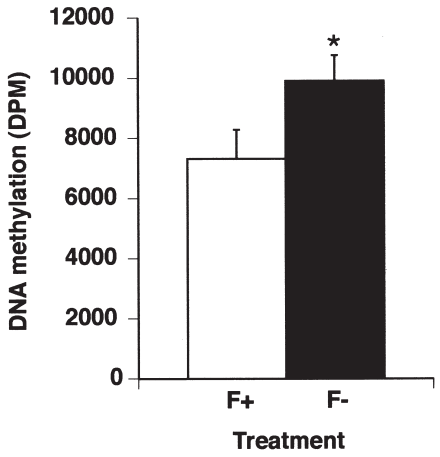

Figure 2. Effect of folate deficiency on genomic DNA methylation in HCEC. HCEC were grown in presence (F+) or absence (F–) of folic acid (4 mg/l) for 14 days. Values (means ± SEM; $n = 8$) are expressed as disintegrations per minute (DPM) per 0.5 µg of DNA. *, $p < 0.005$ vs. presence of folic acid. A higher DPM count reflects a lower degree of DNA methylation.

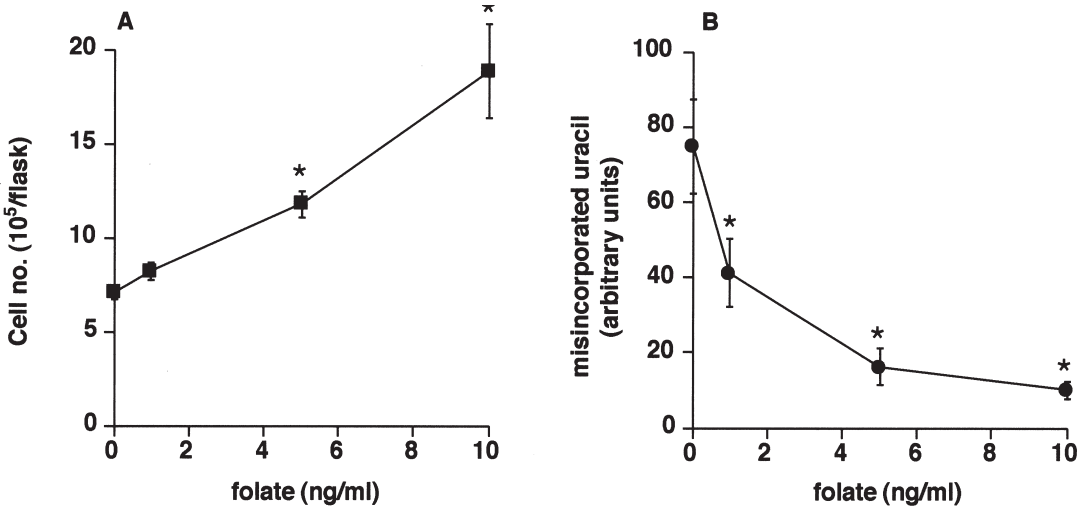

Figure 3. Folate deficiency-mediated inhibition of HCEC proliferation (A) and uracil misincorporation (B) is concentration dependent. HCEC were grown with various concentrations of folic acid for 14 days. Values are means ± SEM; $n = 8$. *, $p < 0.05$ vs. absence of folic acid.

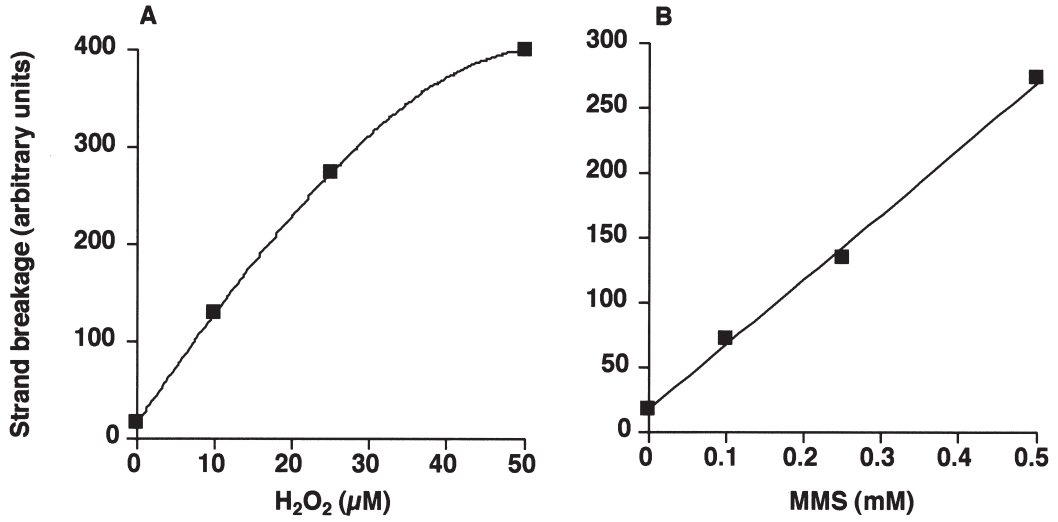

Figure 4. DNA strand breakage in HCEC mediated by H_2O_2 (A) and methyl methanesulfonate (MMS; B). Values are means ± SEM; $n = 8$.

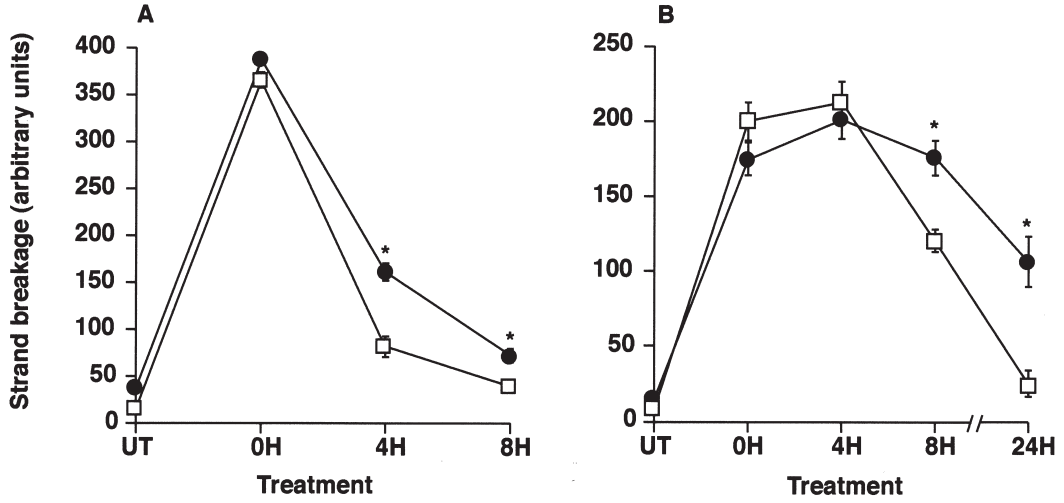

Figure 5. Effect of folate depletion on HCEC repair capacity. HCEC were grown in presence (*open squares*) or absence (*filled squares*) of folic acid (4 mg/l) for 14 days. DNA strand breakage was measured immediately (0 h) or 4, 8, and 24 h after exposure to H_2O_2 (A) or MMS (B). Values are means ± SEM; $n > 8$. *, $p < 0.05$ vs. presence of folic acid.

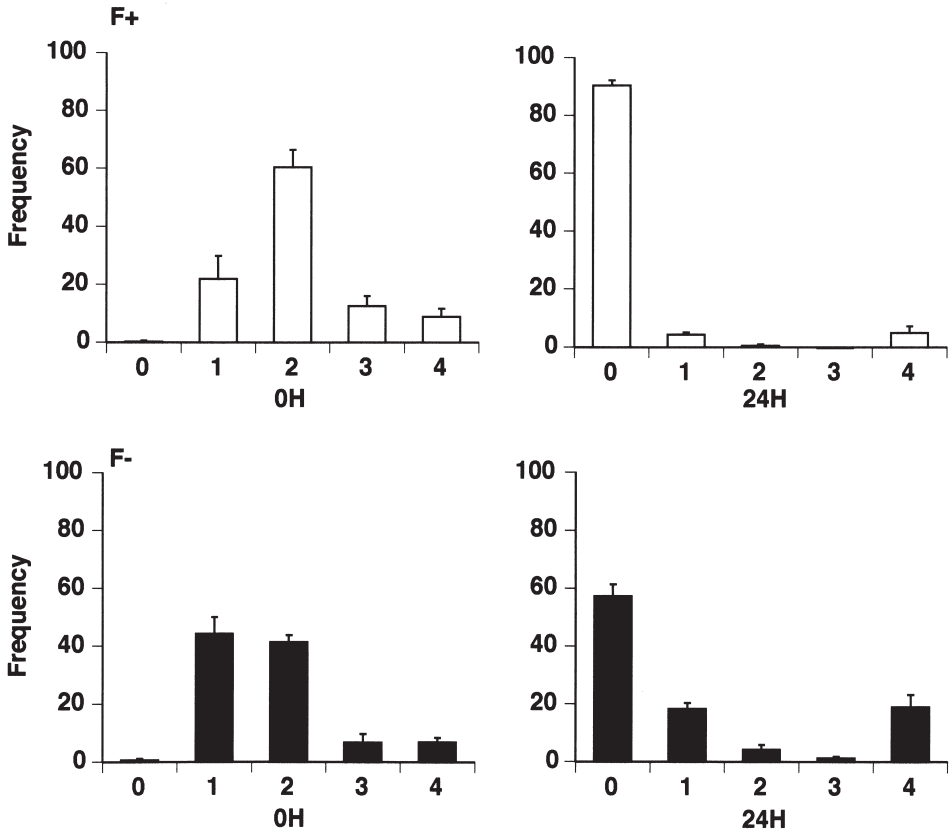

Figure 6. Folate deficiency inhibits DNA repair in response to MMS. MMS-induced DNA strand breakage (0 and 24 h after repair) in folate-replete or folate-deficient HCEC is expressed as frequency of visual classes measured using comet assay. Values are means ± SEM; $n = 8$.

were unable to efficiently remove the MMS-mediated DNA damage compared with folate-sufficient cells [87.4% ± 4.3% ($n > 8$) in control cells vs. 39.1% ($n = 8$) in folate-deficient cells, with repair capacity calculated using the data from Figure 5 and expressed as percent removal of strand breakage over the experimental period].

Discussion

The data linking dietary folate intake with cancer of the colorectum are convincing. Epidemiological studies have reported an inverse association with dietary folate intake and colorectal cancer risk (4–6), whereas intervention with folate decreases the subsequent risk of neoplasia in patients with inflammatory bowel disease (8). Folate deficiency increases DNA instability *in vitro*, in the form of higher DNA strand breakage, chromosomal damage, and mutation frequency (11,28,30).

Folate deficiency may increase malignant transformation by inducing an imbalance in deoxyribonucleotide pools, leading to altered DNA synthesis and inhibited repair (10,11, 28). Folate is essential in the conversion of deoxyuridine monophosphate to thymidine monophosphate, which is a substrate for normal DNA synthesis and repair. By blocking the methylation of deoxyuridine monophosphate to thymidine monophosphate, folate deficiency can disrupt the balance of deoxynucleotide triphosphosphates (dNTPs) in the DNA precursor pool, leading to uracil misincorporation into DNA in place of thymine (11). Sustained folate deficiency may induce a "catastrophic" or "futile" repair cycle of uracil misincorporation and removal, resulting in DNA strand breakage, chromosomal damage, and cancer (11,29). Folate deficiency increases uracil and decreases thymidine levels in HL60 human myeloid cells (31). Similarly, folate deficiency induces DNA strand breakage and uracil misincorporation in isolated human lymphocytes (26). However, human colonocytes are a more valid and representative model for determining how folate status may modulate colorectal cancer.

In this study, we have shown that folate deficiency *in vitro* increases uracil misincorporation in normal human colonocytes. Uracil misincorporation is inversely related to folic acid concentration. Moreover, the concentrations of folate investigated in these experiments are nutritionally relevant (32), suggesting that plasma folate levels, indicative of a diet adequate for the prevention of overt deficiency, may not be optimal in maintaining DNA stability.

Because of its ability to convert homocysteine to methionine, folate is important in the biosynthesis of SAM (9), which in turn regulates gene expression. Folate deficiency may, therefore, decrease DNA methylation (hypomethylation), leading to activation of protooncogenes and induction of cancer. In support of this, SAM levels are dramatically reduced in rodents maintained on a methyl- or a folate/methyl-deficient diet, whereas DNA methylation is decreased and

tumorigenesis is induced (33,34). In addition, DNA hypomethylation is frequently found in human colonic neoplasms (35,36). In this experiment, DNA extracted from folate-deficient human colon cells was hypomethylated.

In addition to negatively affecting DNA synthesis, folate deficiency-mediated alterations in dNTP pools may inhibit DNA repair systems, which likewise require a precise balance of precursors (37). Oxidative damage to DNA, caused by reactive oxygen species, has been implicated in the development of cancer (38). Similarly, DNA alkylation after exposure to ionizing radiation or endogenous/exogenous alkylating agents may induce mutagenesis (39). In this study, human colonocytes grown in folate-free media were unable to effectively repair oxidative- or alkylation-induced DNA damage. This type of damage is removed by the excision repair pathway, which replaces damaged bases with appropriate DNA bases by use of the complementary strand as template. Perturbations in the dNTP pool could result in a breakdown in DNA repair. Folate deficiency significantly reduces DNA excision repair in rodent colonocytes and human lymphocytes exposed to H_2O_2 *ex vivo* (26,40) and inhibits DNA repair *in vitro* in response to radiation in Chinese hamster ovary cells (41). Folate status may also be associated with efficiency of DNA mismatch repair (42). Errors during DNA replication or recombination are corrected by the DNA mismatch repair system. An inverse (but non-significant) association between colonic folate concentrations in patients with inflammatory bowel disease and DNA microsatellite instability as an indicator of efficient mismatch repair has recently been reported (42). Microsatellites are repetitive DNA sequences dispersed throughout the genome. Instability within these sequences is regarded as a biomarker for altered DNA repair (42). Ulcerative colitis patients were shown to have an increased rate of microsatellite instability (13%) and 30–50% lower serum, whole blood, and colonic folate concentrations. Moreover, folate supplementation reduced to normal microsatellite instability in one of the volunteers (42).

A diet poor in folic acid will probably also be deficient in other protective phytochemicals, including vitamin E, carotenoids, and vitamin C, which safeguard cellular components, such as DNA and membranes, from attack from oxidizing and alkylating agents. Compromised antioxidant status, together with folate deficiency-mediated DNA instability and reduced DNA repair, may contribute to the increased risk of colorectal cancer associated with diets low in vegetables and fruit.

In conclusion, folate deficiency *in vitro* induces DNA instability and hypomethylation in immortalized human colonocytes. These data show that poor folate status in these cells can influence uracil misincorporation and DNA methylation status, both of which have been implicated as risk factors for colorectal cancer.

Acknowledgments and Notes

This work is supported by the World Cancer Research Fund and the Scottish Executive Rural Affairs Department. Address correspondence to Dr. Susan J. Duthie, Rowett Research Institute, Greenburn Rd., Bucksburn, Aberdeen AB21 9SB, UK. Phone: +44 1224 712751 ext. 2324. FAX: +44 1224 716629. E-mail: sd@rri.sari.ac.uk.

Submitted 5 January 2000; accepted in final form 30 March 2000.

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