Effect of folate supplementation on mucosal cell proliferation in high risk patients for colon cancer

K Khosraviani, H P Weir, P Hamilton, J Moorehead, K Williamson

Aims: Intracellular folate deficiency has been implicated in colonic carcinogenesis in epidemiological studies and animal and human cancer models. Our aim was to determine the effect of folate supplementation on patients with recurrent adenomatous polyps using rectal mucosal cell proliferation as a biomarker.

Patients and methods: Eleven patients with recurrent adenomatous polyps of the colon were randomised into a treatment group (n = 6) receiving a dietary supplement of 2 mg folic acid per day for three months and a control group (n = 5) receiving a placebo. Rectal biopsies were taken at 10 cm from the anal verge prior to supplementation and repeated at four, 12, and 18 weeks from the start of the supplementation. Each biopsy was immediately incubated in culture medium enriched with bromodeoxyuridine (BrdU). The S phase cells which incorporated BrdU into their DNA were identified following immunohistochemical staining. Twenty five orientated crypts were identified for each time point and the number and position of BrdU positive and BrdU negative cells were counted. BrdU labelling indices (LIs) were calculated for the entire crypt and for each of five equal compartments running consequent from the base to the luminal surface.

Results: The LI of the treatment group (9.1 [6.7, 12.3]) and the control group (9.3 [7.8, 10.3]) were comparable at the start. Over the duration of the supplementation period, LI in the control group did not alter significantly (9.3 [7.8, 10.3] v 9.6 [8.9, 10.4]). However, LI of the folate treated group was lowered after 12 weeks of supplementation (9.1 [6.7, 12.3] v 7.4 [5.3, 9.6]). Analysis of the LI for compartments within the crypt showed that the most significant drop in number of proliferating cells was in the upper most regions of the crypt.

Conclusion: These data indicate that (a) folate supplementation decreases colonic mucosal cell proliferation in a high risk group for colon cancer and (b) the most significant reduction takes place at the luminal aspect of the crypt.

The randomised double blind study described here is the first of its kind to evaluate the effect of dietary supplementation of folate on the proliferative pattern of rectal mucosa in high risk patients with recurrent adenomatous colorectal polyps.

PATIENTS AND METHODS

Twenty patients with recurrent adenomatous polyps of the colon were selected for inclusion in this study. Exclusion criteria were: (1) previous history of CRC; (2) family history of colon cancer or familial polyposis; (3) current metabolic or life threatening disease; (4) recent use of vitamin supplements or non-steroidal anti-inflammatory drugs; (5) pregnancy; and (6) anaemia or B12 deficiency. Patients were randomised into two groups, with 10 in each group (five males, five females). Informed consent was obtained from each patient before inclusion. Ethics approval was granted by Queen’s University of Belfast Research Ethical Committee.

All patients received a 12 week supply of tablets. The test group received 2 mg per day of folic acid whereas the control group received a placebo tablet of sodium chloride. Each patient had rigid sigmoidoscopy performed without previous bowel preparation and four biopsies were taken from the rectal mucosa at 10 cm from the anal verge. This sampling was performed at the outset of the study, at four weeks, 12 weeks.

Abbreviations: BrdU, bromodeoxyuridine; LI, labelling index; CRC, colorectal cancer; PBS, phosphate-buffered saline.
and 18 weeks from the start. One biopsy was sent for histopathological examination and any evidence of mucosal inflammation, atypia, or dysplasia resulted in exclusion from the study.

**Compliance**
Compliance was assessed by pill count. Subjects were provided with three bottles containing the exact number of pills plus three extra.

**Blood sampling**
Blood samples were taken from each patient at each of the four visits and used to measure red blood cell levels of folate. Red blood cell levels of folate were measured as described in the manual with the reagents supplied (SimulTRAC-S Radio-assay Kit; ICN Pharmaceuticals, New York, USA). This work was carried out at the clinical chemistry laboratories in Belfast City Hospital (Lisburn Road, Belfast, UK).

**Dietary questionnaire**
To evaluate any alteration in diet which may have taken place following interview, patients were asked to complete a three day dietary questionnaire at commencement of the study and at the end of the supplementation period. Patients were asked to record their total intake during three separate 24 hour periods, usually two week days and one weekend day. Patients were instructed about the information required—that is, the type and amount of food and drink consumed, and how the food and drink were prepared. From this information and using food analysis (The composition of foods, 5th edition, McCance and Widdowson's) the information was entered into a computer (Microdiet software; University of Salford, Salford, UK) which was able to calculate the total caloric intake, along with breakdown into each component of the food—that is, fat, carbohydrate, proteins, and vitamins with trace elements.

**Bromodeoxyuridine incorporation**
Biopsies were divided into 1 mm pieces and any mucus or faecal residue was detached. Biopsies were orientated under a dissecting microscope onto nitrocellulose paper with the velvety side facing upwards so that the submucosa was next to the paper with the crypts uppermost. They were immediately placed in a wire basket and suspended above a gas filter bubbling 95% O₂ and 5% CO₂ immersed under 30 ml of Dulbecco’s modified Eagle’s medium with 3 ml of fetal calf serum (10%), 360 µl L-glutamine (200 mM), and 1830 µl of stock bromodeoxyuridine (BrdU, final concentration 1000 µM). Incubation was continued for 60 minutes in a water bath at a constant temperature of 37°C.

Following this, samples were fixed in 70% ethanol for four hours. They were then processed routinely to paraffin wax on an automatic tissue processor. Then, biopsies were carefully embedded individually and orientated with the paper standing on its edge in paraffin wax. Sections (3 µm) were cut from the paraffin blocks at a minimum distance of 40 µm apart and dried overnight at room temperature before immunohistochemical staining.

Sections were dewaxed in xylene and rehydrated through descending concentrations of alcohol. Slides were thoroughly washed in phosphate buffered saline (PBS, pH 7.1). DNA was denatured in 1 M hydrochloric acid at 37°C for 12 minutes to expose the incorporated BrdU in single stranded DNA and inhibit endogenous peroxidase activity.

**Bromodeoxyuridine immunohistochemistry**

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Comparison of dietary patterns between the two groups before and after intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily intake</td>
<td>Control (n=5)</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>1625 (380)</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>67 (7)</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>61 (26)</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>196 (50)</td>
</tr>
<tr>
<td>Folate (µg)</td>
<td>170 (39)</td>
</tr>
</tbody>
</table>

Values are mean (SD).

**Calculation of the labelling index in mucosal samples**
Using direct microscopy, well orientated longitudinally sectioned crypts that could be identified from luminal surface to muscularis mucosae were used for analysis. To facilitate scoring, each crypt was divided at the base into two crypt columns or hemicrypts. Starting at the base of the hemicrypt, cells were numbered up to the luminal surface of the colon to determine the number of cells per hemicrypt and then divided into five equal compartments each containing a fifth of the total number of cells. Twenty five complete crypts (50 hemicrypts) were examined for each patient at each time point. The number and position of BrdU labelled cells in the hemicrypt were recorded. The labelling index (LI) was determined for the whole hemicrypt and for each compartment, by dividing the number of labelled cells by the total cells and multiplying by 100.

Total LI = (labelled cells per hemicrypt/total cells per hemicrypt)×100

Compartment LI = (labelled cells per compartment/total cells per compartment)×100
Total crypt labelling indices (LI) for each patient in the folate supplemented group (from 253 µg/l before (t=0 weeks) to 653 µg/l after treatment (t=12 weeks; p<0.05). The control group showed no change in mean red cell levels of folate (198 µg/l before (t=0 weeks) and 200 µg/l after treatment period (t=12 weeks)).

Dietary questionnaire
Mean daily food intake at the start of the study and at the end of 12 weeks in the two groups were similar and there was no alteration in the intake of folate through dietary means between the two time points (table 1).

Labelling index
While crypt length was not measured, the total number of cells per crypt were counted. The total number of cells per crypt were similar between the controls and folate treated group (60 (6.4) and 63 (7.1), respectively) (mean (SD)). The LI for the two groups was comparable before intervention. After 12 weeks of supplementation there was no significant change in the LI of the control group but there was a significant decrease in the LI of the folate treated group (p=0.044) (table 2). The mean reduction in total LI did reach significance after 12 weeks of supplementation when comparing the treated and control groups (p=0.05, two sample t-test).

When the data were analysed on an individual patient basis it was evident that the LI for the crypt and each individual compartment in the control group did not alter significantly throughout the duration of the study. In the folate treated group, although the overall group LI was significantly lower after 12 weeks of supplementation, the total crypt LI was not decreased to the same extent in each of the patients (fig 1). When individual compartment LIs were examined, there was no change in the LI of compartments at the base of the crypt.
(compartments 1, 2, and 3) whereas the LI of the compartments at the luminal surface of the crypts (compartments 4 and 5) was reduced significantly in the folate treated group after 12 and 18 weeks supplementation ($p=0.006$ and 0.007 (paired $t$ test), respectively) (fig 2, table 3). There was no change in compartmental LIs of the control group. When the mean reduction in total LI of compartments 4 and 5 was analysed, there was again a significant difference between the two groups ($p=0.01$, two sample $t$ test). It was also noted that the LI for the entire crypt and the individual compartments showed a trend towards their pretreatment values after supplementation had been discontinued for six weeks (figs 1, 2).

**DISCUSSION**

This study has demonstrated that folate supplementation modulates the state of proliferative cells in the rectal mucosa. More importantly, it demonstrates that the reduction in cell proliferation is induced mainly at the luminal aspect of the crypt. The presence of labelled cells in the upper crypt is thought to reflect defective cell proliferation control and delayed onset of normal differentiation. In fact, it has been commented that evaluation of the upper most compartments of the crypt provides the most discriminating evidence of high risk. Furthermore, studies in animal models have shown that nutrient induced changes in colonic mucosal crypt cells are directly related to changes in tumour risk. In 1997, Biasco and colleagues demonstrated that folate supplementation regulated rectal mucosal cell proliferation in patients with long standing ulcerative colitis. However, this is the first report of the effect of folate in patients with recurrent adenomatous polyps.

Both the dietary questionnaires and red cell folate results provide evidence that other environmental factors have been controlled as far as possible. This lends support to our finding that the effect seen in the proliferative pattern is produced by folate supplementation. Studies of blood folate levels in relation to colorectal adenomas raise the question as to whether patients with cancer or polyps might have less efficient absorption and/or metabolism of folate than normal. Interestingly, mean red blood cell folate levels of adenoma patients have been reported to be substantially less than those of controls. It is accepted that the usual dietary intake of folate is about 200 µg/day, therefore we used a supraphysiological level of folate (four times the dietary requirement) lead to a degree of $p53$ integrity greater than that observed with basal diet. When human lymphoid cells are treated with inhibitors of folate metabolism, such as methotrexate, folate dependent thymidine synthesis is impaired, leading to substitution of thymidylate by uridylate in DNA synthesis. This may be a mechanism whereby low folate levels lead to the occurrence of missense or non-sense gene mutations.

Folate deficiency may also induce DNA strand breaks which are associated with neoplastic transformation. Supraphysiological levels of folate supplementation (four times the daily dietary requirement) lead to a degree of $p53$ integrity greater than that observed with basal diet. When human lymphoid cells are treated with inhibitors of folate metabolism, such as methotrexate, folate dependent thymidine synthesis is impaired, leading to substitution of thymidylate by uridylate in DNA synthesis. This may be a mechanism whereby low folate levels lead to the occurrence of missense or non-sense gene mutations.

**Table 3** Comparison of the labelling index (LI) (compartments 4 and 5) in the two treatment groups over the four time points (supplementation was given only for the first 12 weeks)

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (weeks)</th>
<th>0</th>
<th>4</th>
<th>12</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>3.1 (1.4, 4.7)</td>
<td>2.4 (1.5, 3.3)</td>
<td>2.6 (2.0, 3.2)</td>
<td>2.0 (1.5, 2.5)</td>
<td></td>
</tr>
<tr>
<td>Folate</td>
<td>3.9 (2.1, 5.7)</td>
<td>2.6 (0.7, 4.5)</td>
<td>0.6 (0, 1.6)*</td>
<td>1.5 (0.6, 2.4)**</td>
<td></td>
</tr>
</tbody>
</table>

* $p=0.006$, ** $p=0.007$ (paired $t$ test)

Values are mean patient LI (compartments 4 and 5) (95% confidence limits).

Folate deficiency may also induce DNA repair in the colonic mucosa. In male Sprague-Dawley rats, folate deficiency reduces DNA excision repair. It does not itself affect mismatch repair, as measured by microsatellite instability. The established rat colon procarcinogen 1,2-dimethylhydrazine does not induce microsatellite instability on its own but does induce this when animals are folate deficient. Thus folate deficiency may diminish multiple aspects of DNA repair in the colonic mucosa.

Another mechanism of action may involve activation of protein kinase C, which is associated with CRC in humans and animals. This may occur because of a deficiency in choline which, like methionine, is a methyl donor. In cases of low term folate deficiency, choline levels are also reduced.

Folate deficiency may also lead to chromosomal abnormalities at fragile sites. CRC has been associated with disruption of chromosomal integrity with loss of tumour suppressor gene activity. It may also lead to impaired ability of natural killer cells to destroy dysplastic or neoplastic cells.
In conclusion, these data provide evidence that folate supplementation can regulate colonic mucosal cell proliferation in patients with recurrent adenomatous polyps, as a subgroup at high risk of colon cancer. The reduction in the proliferative cell numbers is most significant at the upper zones of the crypts. These data also indicate that the effect of the supplementation may persist for a period after the supplement is discontinued. This report highlights the need for further investigation on the role of folate as a chemopreventive agent in patients at risk of colon cancer.

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REFERENCES
27 Lipkin M. Biomarkers of increased susceptibility to gastrointestinal cancer. Gastroenterology 1987;92:1083–6.
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