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

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**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 consequentially 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.
Biopsies were divided into 1 mm
Bromodeoxyuridine incorporation
UK) which was able to calculate the total calorific intake,
McCance and Widdowson’s) the information was entered into
and using food analysis (how the food and drink were prepared. From this information
that is, the type and amount of food and drink consumed, and
hour periods, usually two week days and one weekend day.
asked to record their total intake during three separate 24
day dietary questionnaire at commencement of the study and
following interview, patients were asked to complete a three
To evaluate any alteration in diet which may have taken place
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
hours 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 calorific 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 mm² 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 bub-
bbling 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 bromode-
xyuridine (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 immuno-
histochemical 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
Primary antibody solution (100 µl) containing 4 µl human
serum and 2 µl mouse anti-BrdU (Bu20a) monoclonal
antibody (Dako M744, Bucks, UK) diluted in 1:50 PBS with
0.05% Tween 20 (Sigma P1379) was applied to each slide and
incubated for 60 minutes at room temperature. This was
followed by 100 µl of biotinylated secondary antibody
solution, containing 4 µl human serum and 0.5 µl biotinylated
rabbit anti-mouse F(ab’), antibody (Dako E413) diluted to
1:200 in PBS with Tween, for a further 60 minutes. Slides
were rinsed in PBS before 100 µl streptavidin-biotin-peroxidase
complex (Dako K 377) was applied for 30 minutes. Antibody
binding was visualised with the chromogen diaminobenzidine
(Sigma, Dorset, UK), primed with 100 µl of 30% H₂O₂. Finally,
the sections were lightly counterstained with Harris haema-
toxylin (Sigma HHS-128) for 10 seconds to allow the
non-labelled nuclei to be visualised and counted. As a negative
control, sections were incubated with a class matched
non-specific mouse Ig monoclonal antibody instead of the
primary Bu20a monoclonal antibody. As a positive control,
a section known to stain positively was included in each run.

Calculation of the labelling index in mucosal samples
Using direct microscopy, well orientated longitudinally sec-
tioned 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.

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Total\ LI = \frac{\text{labelled cells per hemicrypt}}{\text{total cells per hemicrypt}} \times 100
\]

\[
\text{Compartment LI} = \frac{\text{labelled cells per compartment}}{\text{total cells per compartment}} \times 100
\]
Statistical analysis

All LI data obtained throughout the study displayed a normal distribution. On this basis, a paired Student’s t test was used to identify differences between baseline LIs and LIs at 12 weeks in the control and folate groups separately. A two sample t test was utilised to detect differences in mean reduction over the supplementation period between the treated and control groups. Due to the small sample size, the analysis was also performed using Wilcoxon’s signed rank test for comparison between groups and the Mann-Whitney test for paired analysis to corroborate the findings.

The results of red blood cell levels of folate and dietary nutrient intake were analysed using a non-parametric method (Mann-Whitney U test). In all cases, results were considered to be significant when p<0.05.

RESULTS

Both groups were similar with respect to sex distribution, age, and weight.

Compliance

Only 11 patients completed the trial; the main reason for non-compliance was poor tolerance for repeated rigid sigmoidoscopy. All patients returned three bottles which contained the supplements. In only two cases was there less than three pills remaining in each bottle.

Blood sampling

As expected, there was a marked increase in mean red cell folate levels 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.
subjects with advanced malignancy. In these patients increased folate levels are believed to increase malignant cell turnover. There is also a concern that subjects who are receiving antiepileptic medication may need to alter the dosage of their drugs during folate supplementation. However, there is no evidence that folate supplementation interferes with the efficiency of antifolate chemotherapeutic agents. Timing of any dietary supplementation is also an important consideration because this may influence whether the effects suppress or enhance tumorigenesis.

There are a number of hypotheses to explain the putative relationship between low intake of folate and colon cancer, which may be reversed by increasing total body folate level by dietary means or supplementation. These include: (a) induction of DNA hypomethylation; (b) induction of DNA damage; (c) impairment of DNA repair; (d) secondary depletion of choline; and (e) chromosomal abnormalities at fragile sites.

In the presence of low levels of folate, DNA hypomethylation may be induced in colorectal neoplasia and occurs as a global phenomenon. DNA hypomethylation is noted to increase from mucosa in normal subjects to normal mucosa in subjects with neoplastic lesion, to adenomas, and finally carcinoma. DNA hypermethylation precedes most of the other molecular events that occur in colorectal carcinogenesis, suggesting that alterations in DNA methylation are aetiologically important.

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.

Folate deficiency may impair 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.

Folate deficiency may also contribute 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.

### 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 suprapharmacological dose of 2 mg/day to achieve a significant increase in body folate levels, as shown by the red cell measurements. There is a report that increased dietary folate of up to four times the basal requirement in an animal model demonstrates a beneficial effect in reducing macroscopic tumour load.

Interestingly, it was found that dietary means of increasing daily folate intake was only related to a modest reduction in the risk of colon cancer while the reduction was more pronounced with specific folate supplements. Conversely, there are risks associated with folate supplementation, especially in patients with B12 deficiency, and interestingly in

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

*p=0.006, **p=0.007 (paired t test)
Values are mean patient LI (compartments 4 and 5) (95% confidence limits).
In conclusion, these data provide evidence that folate supplementation can regulate colonic mucosa 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.

ACKNOWLEDGEMENTS

This work was supported by Research and Development Grant (DHSS), Royal College of Surgeons of Edinburgh, and Action Cancer (Northern Ireland).

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