Influence of diets containing high and low risk factors for colon cancer on early stages of carcinogenesis in human flora-associated (HFA) rats

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Abstract: Germ-free rats colonised with a human intestinal flora were fed diets containing high risk (HR) or low risk (LR) factors for colorectal cancer, and putative biomarkers were evaluated in the colonic mucosa; (i) proliferation, (ii) 1,2-dimethylhydrazine (DMH)-induced aberrant crypt foci and (iii) DMH-induced DNA damage. The HR diet was high in fat (45% of calories) and low in calcium and fibre, reflecting levels characteristic of typical western diets. The LR diet was low in fat (<5% of calories), and high in calcium and fibre. The nutrient/energy ratio of the two diets were similar. Mucosal crypt cell proliferation, assessed after microdissection, was higher on the LR diet (mean number of mitoses per crypt was 2.65 on the LR diet, and 1.62 on the HR diet; \( P < 0.05 \)). Aberrant crypt foci (ACF) were assessed in the mucosa 12 weeks after DMH treatment. On the HR diet there were significantly more small ACF with 1 and 2 crypts per focus, but fewer ACF with 3, 5 and 7 or more crypts per focus. There was no significant difference in total ACF or the total number of crypts. The effect of diet on DNA damage in the colon was assessed \textit{in vivo} by the comet assay. Animals were fed a HR or LR diet for 12 weeks before treatment with DMH or saline. For carcinogen-treated animals, DNA damage was significantly higher in colon cells from animals on the HR diet. On the LR diet both DNA damage and the induction of small ACF were reduced despite an increase in cell proliferation. The increase in large ACF on the LR diet may be attributable to elevated crypt cell proliferation possibly increasing crypt fission rates.

Introduction

The aim of this study was to assess the impact of diets containing either high risk or low risk factors for colorectal cancer on the range and variation of a number of supposed precancer events used to assess dietary risks for cancer in an experimental model of the rat colon. Dietary fat, particularly animal fat, has been strongly correlated with the incidence of colon cancer (1), whilst low fat, high fibre and high calcium diets, as well as diets with a high vegetable or fruit content, appear to be protective (2). Epidemiological studies are extremely costly and time consuming and, whilst molecular epidemiological studies can provide information about the process of carcinogenesis, the use of animal models allows more drastic intervention which can be used to evaluate potential risk factors and biomarkers associated with colon cancer.

Because of the purported role of the human intestinal microflora in the aetiology of colon cancer (3,4) the animal model used in the study comprised germ-free rats colonised with a human faecal microflora (human flora-associated, HFA*, rats). These rats provide a particularly relevant model of the human colon since intestinal bacteria are maintained in proportions similar to that of the human donor. Alterations to the diet can have marked effects on microbial activity in the colon, and for dietary studies the extensive and varied microbial metabolism is more accurately reflected by HFA than conventional rats (5,6).

The high risk (HR) diet was designed to represent the high-fat, low-fibre diet typical of western populations with a high incidence of colorectal cancer. The HR diet was also low in calcium, since high calcium diets may protect against colon cancer (7). The low risk (LR) diet was low in fat (5% of calories), and was high in fibre and calcium.

Aberrant crypt foci (enlarged, hyperplastic colonic lesions, ACF) are considered to be preneoplastic precursors for carcinogen induced tumours of the colon in experimental rats (8–10) and mice (11,12), and for colorectal adenocarcinomas in humans (13). It has been proposed that the total number, distribution or multiplicity of ACF correlates with the incidence of colorectal tumours (8,11,12,14); although this relationship does not appear to be a straightforward one (14,15), and is likely to be dependent on whether dietary factors affect the induction or the promotion of ACF. ACF induced using DMH were used to test the modulatory effects of complex diets containing high-risk and low-risk factors for colorectal cancer. DMH is a complete carcinogen able to induce tumours in the small and large intestine with a high degree of selectivity (16), and is a potent inducer of ACF (10). DMH has also been shown to induce DNA fragmentation (single strand breaks) in the DNA of both the liver and lung of mice (17).

Hyperproliferation of the colonic epithelium is considered to be a biomarker of an increased susceptibility to colon cancer (18), and diets with antiproliferative effects on the colonic epithelium are thought to be beneficial (19,20). There are, however, a number of dietary components, such as dietary fibre, which increase colonic cellular proliferation and so may inversely correlate with colon cancer risk (21) and the induction of ACF (22).

The third biomarker for colon cancer assessed in the study was damage to the DNA of colonic mucosa cells. DNA damage is a well accepted initial step in mutagenesis, and recent developments in the single-cell electrophoresis (comet) assay allow quantitation of DNA damage in single cells isolated from the intact colonic epithelium (23,24). The comet assay provides a simple, sensitive and accurate assessment of DNA damage in isolated cells (25). By using the alkaline lysis method single strand breaks (SSB’s) can be detected which

*Abbreviations: HR, high risk; LR, low risk; DMH, 1,2-dimethylhydrazine; ACF, aberrant crypt foci; HFA, human flora-associated; SSB’s, single strand breaks; MR, medium risk, HBSS, Hank’s balanced salt solution.
At 15–17 weeks of age all animals were fed a medium risk (MR) diet. Diet and carcinogen administration

In Table I, the formulation of high risk (HR) and low risk (LR) diets (g per kg diet) is shown. The ingredients are as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>HR</th>
<th>LR</th>
<th>MR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>231</td>
<td>716</td>
<td>473.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>231</td>
<td>0</td>
<td>115.5</td>
</tr>
<tr>
<td>Sodium caseinate</td>
<td>254</td>
<td>200</td>
<td>227</td>
</tr>
<tr>
<td>Lard</td>
<td>204</td>
<td>0</td>
<td>102</td>
</tr>
<tr>
<td>Corn oil</td>
<td>26</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>AIN vitamin mix</td>
<td>12</td>
<td>9.3</td>
<td>10.65</td>
</tr>
<tr>
<td>AIN minerals (low Ca²⁺)</td>
<td>40</td>
<td>30.9</td>
<td>35.45</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2</td>
<td>1.5</td>
<td>1.75</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>0</td>
<td>22.5</td>
<td>11.25</td>
</tr>
</tbody>
</table>

Fibre

- Wheat bran            | 0   | 50  | 25   |
- Solka flok            | 10  | 50  | 30   |
- Inulin               | 0   | 50  | 25   |

Total energy (kJ) 20,220 15,596 17,908

A small 0.5 cm² biopsy was taken from the distal colon and immediately fixed for 30 min in Carnoy’s fluid (60% ethanol, 30% chloroform, 10% acetic acid by volume). Biopsy samples were then transferred to absolute ethanol and microdissected within 1 week. For each animal (15 per group) the number of mitotic cells in 20 crypts were counted (29).

Comet assay

Colonocytes were isolated by protease digestion according to previous methods (28). Body weight increased by 23% on the HR diet and only 15% on the LR diet. There was no significant difference between the head and tail means) (26). When the number of breaks increases, more DNA can migrate under the influence of an electric current to form a tail where it can be detected and measured as a tail moment (product of the percentage of DNA in the tail distribution and the displacement between the head and tail means) (26).

Microdissection and mitotic index counts of normal crypts

A small 0.5 cm² biopsy was taken from the distal colon and immediately fixed for 30 min in Carnoy’s fluid (60% ethanol, 30% chloroform, 10% acetic acid by volume). Biopsy samples were then transferred to absolute ethanol and microdissected within 1 week. For each animal (15 per group) the number of mitotic cells in 20 crypts were counted (29).

Comet assay

Colonocytes were isolated by protease digestion according to previous methods (28). Briefly, the intact distal colon was excised and rinsed with Hank’s balanced salt solution (HBSS) to remove any faecal material. The colon was then filled with 40 mM dithiothreitol in HBSS, the ends of the colon ligated and left at 20°C for 10 min to dispel mucus. Colonocytes were liberated by enzymatic digestion (3.7 mg ml⁻¹ in Ca²⁺⁺- and Mg²⁺⁺-free HBSS) at 37°C for 30 min. After removal of any contaminant mucous, cells were collected after centrifugation at 200 × g for 10 min and resuspended at 2×10⁶ cells ml⁻¹ in RPMI-1640 medium. Cell viability was assessed using trypan blue, and 2×10⁶ viable cells were suspended in 0.5% low-melting-point agarose at 37°C on a fully foiled, agarose-coated microscope slide. The slides were cooled and, once the agarose had set, were overlaid by a second agarose layer. Colonocytes were then counted using a Zeiss stereo microscope at 40× magnification.

Materials and methods

Human flora-associated (HFA) rats

Twenty-four male and 14 female F344 rats (30 for ACF determination and 8 for comet analysis) born germ-free and weaned at 3 weeks, were used. All animals were initially fed a stock rodent diet (R and M No.1, Special Diet Services, Witham, Essex, UK) which had been sterilised by γ-irradiation from value that gave the most appropriate representation of the distribution of taila 60 Co source (50 Gy). All the 75th percentile value for tail moment was used to calculate the mean value.

Statistics

The 75th percentile value for tail moment was calculated from the tail moments of 50 cells per slide, 3 slides per animal, and provided a single value that gave the most appropriate representation of the distribution of tail moment data. For tail moments and mitotic index evaluation a nested ANOVA and least significant difference test was conducted using GenStat 5 software. ACF were evaluated using the Mann–Whitney two-sample rank test (Minitab, Inc., PA, USA).

Results

Food intake and body weight

The HR diet was of higher energy density than the LR diet, but was designed to have a similar nutrient/energy ratio. Whilst there was a significant increase in the weight of food consumed by animals on the LR diet, there was no significant difference in overall energy intake by the two groups of rats (Table II). Animals on the HR diet gained more weight (P < 0.01 for males; P < 0.05 for females) than those on the LR diet; male body wt increased by 23% on the HR diet and only 15% on the LR diet.
Table II. Food intake, body weight gain and weights at post mortem

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
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<tbody>
<tr>
<td></td>
<td>LR diet</td>
<td>HR diet</td>
</tr>
<tr>
<td>Mean daily intake (g)</td>
<td>24.7 ± 3.6</td>
<td>17.0 ± 3.4***</td>
</tr>
<tr>
<td>Mean daily intake (kJ)</td>
<td>348.6 ± 67.0</td>
<td>332.8 ± 57.5</td>
</tr>
<tr>
<td>Initial body wts (g)</td>
<td>340.6 ± 14.5</td>
<td>349.4 ± 11.2</td>
</tr>
<tr>
<td>Final body wts (g)</td>
<td>383.0 ± 14.8</td>
<td>419.6 ± 9.2***</td>
</tr>
<tr>
<td>Mean body wt gain (g)</td>
<td>50.0 ± 6.0</td>
<td>80.0 ± 7.1**</td>
</tr>
</tbody>
</table>

Results shown are means ± 1 SD. Males n = 8 per group, Females n = 7 per group. Values marked with asterisks differ significantly from LR diet (*P < 0.05; **P < 0.01; ***P < 0.001).

Fig. 2. Distribution of aberrant crypts and ACF induced 12 weeks after DMH treatment in HFA rats. Values are the means from 15 animals ± the standard error of the mean (SEM). Significantly different to HR diet (*P < 0.05; **P < 0.01).

for DMH-treated rats there were major differences between the dietary groups in the distribution of tail moments (Figure 3a). Undamaged cells comprised 60.7% of the total in DMH-treated LR rats compared with only 32.7% in HR rats. In the highest category of damage (>30 units) the corresponding values were 9.7% for LR and 26.3% for HR. Upper quartile values (Figure 3b) highlighted a significant increase in DNA strand breaks in cells isolated from the distal colons of rats fed the HR diet compared with those on the LR diet (P < 0.01).

For saline-treated animals, there was a greater proportion of undamaged cells on the LR (95%) than on the HR diet (82.7%) and a reduced number of damaged or severely damaged cells (only 1.0% on the LR compared with 10.3% on the HR diet; Figure 3a). Whilst the change in the distribution of tail moments appeared to reflect a greater degree of damage on the HR diet for non-carcinogen treated controls, the change
in 75th percentile tail moment values was not statistically significant (Figure 3b).

**Discussion**

The aim of this study was to determine the effects of diets with a high and low risk for colon cancer on putative biomarkers in human flora-associated rats; specifically the induction of ACF, changes in proliferation in the colonic crypts and, as a separate and novel biomarker, whether diet could protect against the induction of DNA damage in the colon.

There was no difference due to diet on the total number of ACF induced or on the total number of crypts (crypt multiplicity). This suggests that the overall induction and progression rates were the same for both diets. However, when individual crypt multiplicity is examined there was a significant decrease in the number of small ACF with 1 and 2 crypts per focus, and a significant increase in the number of large ACF with 3, 5 and 7 or more crypts per focus on the LR diet (Figure 2), when compared with the HR diet. Also, crypt cell proliferation was increased and DNA damage decreased in animals receiving the LR diet.

Previous reports have associated reductions in the numbers of small ACF with a decrease in the proliferation rate, and have suggested that the lower turnover protects cells from the effects of DNA damage (30,31); as a low proliferation rate allows more time for repair. This assumption does not apply in the current study where, on the LR diet, an increase in the proliferation rate was accompanied by a decrease in the number of small aberrant crypts and an increase in some of the larger crypts. DNM treatment is known to elevate proliferative activity in the colon (30,32) and, although there is evidence that increased cell proliferation per se does not lead to the development of dysplastic crypts (33), colonic mucosal hyperplasia significantly increases the frequency of DNM-induced focal atypia (34). Since the LR diet increases proliferative activity it would also be expected to increase, not reduce, the number of small ACF. The increase in crypt cell proliferation that occurs after DNM treatment, however, is probably sufficient to mask the small, but chronic increase induced by the LR diet. Since the reduction in the numbers of small aberrant crypts did not appear to be due to changes in the proliferation rate, it suggests that the early, DNM-induced events that lead to the formation of ACF were inhibited by another mechanism.

DNA damage is a well accepted, indeed obligate step for initiating carcinogenesis. The comet assay provides a rapid and sensitive technique for detecting DNA breaks in individual cells (25,26,35). Since the production of ACF are dependent on carcinogenic damage (12), results from the comet assay suggest that either (i) the LR diet inhibited the induction of DNA damage by DNM shortly after the DNM was administed, or (ii) on the HR diet significantly more DNA damage was induced in colonic crypts. We can hypothesise that the LR diet contains factors which may protect against the early stages of carcinogenesis (the initiation of DNA damage) but has a lesser role in disease progression. An alternate hypothesis is that factors in the HR diet enhance DNA damage in this model.

On the LR diet, increases in the larger foci may have occurred after long term increases in the proliferation rate effecting an increase in the crypt fission rate (36). It has been shown that in rats treated with DNM the ACF that develop do so due to a fission mechanism (37). The increased proliferation due to DNM treatment is only short-lived (30) and it is therefore feasible that the slight rise in proliferative activity in the crypts could account for a supposed increase in the crypt fission rate; resulting in increased crypt multiplicity.

Others have found that, in certain models, hyperproliferation is an unlikely explanation for increases in DNA damage. Increases in epithelial proliferation and a reduction in small ACF has been shown to occur when wheat bran, which is known to protect against tumorigenesis in the colon (38), is
administered (22.39, and cholic acid also reduced the number of ACF whilst exerting a hyperproliferative response (40).

The current study has shown that diet may have an effect during the early stages of the carcinogenic process; protecting or exposing cells to DNA damaging agents. Increases in proliferation in the colon may not be as deleterious as previously thought, since both DNA damage and the induction of small ACF are both reduced despite an increase in cell turnover. Proliferation may also complicate the interpretation of ACF data, since increases in crypt multiplicity may not be related to carcinogenic potential but simply reflect increases in crypt fission rates expanding non-tumorigenic aberrant crypts.

Acknowledgements

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References