Dietary n-3 PUFA increases the apoptotic response to 1,2-dimethylhydrazine, reduces mitosis and suppresses the induction of carcinogenesis in the rat colon

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Introduction

Both epidemiological and experimental studies suggest that colorectal cancer is strongly influenced by nutritional factors, including the quantity and composition of dietary fat (1). Diets containing high levels of marine fish oils rich in the n-3 polyunsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA; c20:5) and docosahexaenoic acid (DHA; c22:6) are protective in animal models (2–4) and apparently in human populations (5,6), but the precise mechanisms by which these PUFA exert their anti-carcinogenic effects is unclear. Previous studies have established that fish oil inhibits the initiation, post-initiation and promotion stages of carcinogenesis in rodents following administration of the colon carcinogens 1,2-dimethylhydrazine (DMH) and azoxymethane (AOM) (3,4). Modulation of epithelial cell turnover in the colonic crypt may be an important mechanism in the protection afforded against DMH/AOM-induced carcinogenesis by fish oil. Recently, consumption of fish oil has been shown to attenuate the activities of a number of biochemical pathways implicated in the aberrant regulation of proliferation and cell death during tumorigenesis (7–9).

DMH- and AOM-induced tumorigenesis in the rat colon is a prolonged multistage process, bearing many of the same cell kinetic, histopathological and molecular characteristics of tumorigenesis in the human colon. In the rat, exposure to carcinogen rapidly suppresses proliferation and induces high levels of apoptosis within the colonic crypt (10). This acute damage is followed by a phase of compensatory responses, which restore the mucosa to an apparently normal state within 4–5 days of the injection (11). Target cells for the mutagenic action of DMH/AOM which escape apoptotic deletion from the crypt during this period may have the potential to give rise to tumours after a prolonged period of latency (12). It is probable that factors which can heighten the tendency for DNA-damaged cells to undergo apoptosis following carcinogenic insult have the potential to reduce the incidence of tumours in the colon. Recent evidence suggests that the inherent capacity of colonic crypt cells to undergo apoptosis in response to damage induced by DMH is open to modulation by dietary factors. For example, food deprivation increases apoptotic cell counts induced by DMH (13) and sinigrin, a glucosinolate found in brassica vegetables, increases the frequency of apoptotic cell death in the rodent colonic mucosa for up to 72 h after injection of the carcinogen (14).

Previous studies have indicated a capacity for dietary fish oil to modulate cell turnover in the colonic mucosa of normal rats (15) and humans (16). Recently, it has been reported that prolonged feeding with fish oil increases apoptosis in the colonic mucosa during the promotion stage of chemical carcinogenesis (17, 18), but the stage of carcinogenesis and mechanism by which dietary lipids interact with the induction of neoplasia are largely unknown. In the present study, we manipulated dietary lipid intake during a 48 h period following administration of DMH, and used the incidence of aberrant crypt foci (ACF) as a biological marker of neoplasia (19) to test the hypothesis that dietary fish oil may suppress the induction of neoplastic lesions by modulating the balance of mitosis and apoptosis in the crypt at a critical stage, as it responds to the cytotoxic and genotoxic effects of DMH.

Materials and methods

Animals and diets

Male Wistar rats (125–150 g) were housed individually in wire-bottomed cages, maintained in a temperature- and humidity-controlled animal unit with
daily light:dark cycle of 12 h, and allowed to consume tap water and a basal fibre-free semi-synthetic diet (CO) *ad libitum* for 4 weeks prior to the start of the experiment. The diet contained starch (280 g/kg), sucrose (380 g/kg), casein (200 g/kg), corn oil (80 g/kg) and a balanced mineral and vitamin mix. After administration of DMH, the treatment group was transferred to a diet containing de-acidified bleached fish oil (FO; EPA 18.7%; DHA 8.0%, 96.7% tri-glyceride; Callanish, Breasclete, UK) as the major source of lipid substituted for corn oil (80 g/kg). Food intake was measured daily, and weight gain was recorded weekly and at death. All procedures were approved by the UK Home Office.

**Carcinogen administration and recovery of tissues**

Animals were given either 1,2-dimethylhydrazine dihydrochloride (DMH; Fluka, Gillingham, UK; 30 mg/kg body wt.), in 25 mM EDTA/0.137 M NaCl solution (pH 6.4) by s.c. injection to the scruff of the neck, or a sham injection of EDTA/NaCl carrier. At the end of the experiment rats were killed by i.p. injection of 1 ml (200 mg/ml) pentobarbitone sodium (Euthatal; Rhone Merieux, Harlow, UK) followed by cervical dislocation. The abdomen was opened by a midline incision, the colon was removed intact by excision at the caeco-colonic junction and at the pelvis, flushed with phosphate buffered saline (pH 7.4), and its length was recorded.

**Experimental design**

**Experiment 1.** To assess the influence of dietary PUFA on apoptosis and mitosis in the colon 24 and 48 h after DMH, 40 male Wistar rats were assigned randomly into four groups (CO, FO, DMH-CO, DMH-FO). Rats assigned to the CO and FO groups received a sham injection of EDTA/NaCl carrier and were transferred to the CO or FO diet accordingly. Rats assigned to the DMH-CO and DMH-FO groups were given DMH, and then fed either the CO or FO diet. Dietary changes were executed within 30 min of carcinogen administration. Five rats from each group were killed after a further 24 and 48 h, and their large intestines removed. The colon was everted on a 3 mm diameter metal rod, cut in half, and tissue samples (~5 mm long) were excised from the distal and mid-colon, flanking the distal segment of the colon. Samples were fixed and stained, and the frequency and distribution of crypt cell apoptosis and proliferation were analysed as described below.

**Experiment 2.** To assess the effects of manipulating dietary lipid intake on the induction of ACF, 28 male Wistar rats were assigned randomly to four treatment groups (*n* = 7), and given three injections (7 days apart) of DMH or EDTA/NaCl carrier. Following each injection, animals either remained on the CO diet or were fed the FO diet for 48 h and then returned to the CO diet thereafter. Multiple injections were given to ensure a high yield of ACF, and they were spaced at weekly intervals to enable time for incorporation and clearance of the test lipid between carcinogen treatments. After the final injection all animals were fed the CO diet and housed as described for 18 weeks, killed and their colons removed. Tissue samples for analysis of apoptosis and proliferation were collected as in experiment 1. In addition, the distal half of the colon was cut longitudinally along its length and fixed flat in ethanol:acetic acid (75:25) overnight between filter paper. The filter paper was removed, the tissue stained (as described below) and subsequently laid flat on a microscope slide. Under a light microscope (magnification ×100) the mucosal surface of the distal colon was scanned for aberrant crypt foci (ACF), and the frequency and crypt multiplicity of foci recorded. ACF were identified as elevated focal lesions having one to multiple abnormal crypts with thickened epithelial lining and enlarged luminal openings compared with the normal adjacent mucosa (14).

**Analysis of crypt cell apoptosis and proliferation**

The frequency and spatial distribution of apoptotic and mitotic cells within intact microdissected crypts were determined using morphological criteria (14). Briefly, samples fixed in ethanol:acetic acid (75:25) were rehydrated in distilled water, hydrolysed in 1 M HCl for 7 min at 60°C, stained with Fuellgen’s reagent for 30 min (15 mM basic fuschin, 45 mM potassium metabisulphite, 5% 1 N HCl in distilled water) and stored in 45% acetic acid. Under a standard dissection microscope, the muscle layers were teased from the mucosa and thin strips of crypts micro-dissected from the tissue using a fine gauge needle. The strips were then lightly compressed under a coverslip to separate and flatten the crypts, and 20 randomly chosen intact crypts per animal (20 crypts/group) were viewed under a light microscope (magnification ×400). Apoptotic cells were identified by the presence of condensed chromatin and spherical apoptotic bodies containing nuclear material (Figure 1) as described by Kerr *et al.* (20). Nuclei which were visibly in prophase, metaphase, anaphase, and telophase were classified as mitoses (Figure 2). The length of each crypt was determined by comparison with a calibrated linear eyepiece micrometer (Nikon UK, Kingston, UK), and the positions of mitotic and apoptotic nuclei were recorded and allocated to one of 10 equally spaced longitudinal compartments. Unless otherwise stated data are expressed as mitoses or apoptotic nuclei per crypt.

**Statistical analysis**

Data are expressed as means with standard errors. The significance of differences between groups was assessed by two-way analysis of variance using the general linear model or by one-way analysis of variance coupled with Tukey’s *t*-test for comparison of individual means. All analyses were carried out using the Minitab statistical package (State College, PA).

**Results**

**Food intake and weight gain**

There were no significant differences in food intake and weight gain between dietary groups, and no significant differences in weight gain, weight at death or colon length between animals given DMH or sham injections.

**Effects of carcinogen and lipid on apoptosis**

Apoptosis was negligible (<0.01 apoptotic cells per crypt) in the colonic tissue of animals given a sham injection and fed the CO diet. Treatment with DMH induced an increase in crypt cell apoptosis and the levels observed varied according to the type of dietary lipid (Figure 3). The data for all groups were analysed separately at 24 and 48 h using the general linear model, with DMH treatment, lipid and position in the colon (mid or distal) as factors. At 24 h, the frequency of apoptotic nuclei per crypt was significantly higher in animals...
Fig. 3. Apoptosis in crypts from the distal and mid colon of rats, 24 and 48 h after DMH or sham injection of saline carrier. Apoptosis was negligible (<0.01 cells per crypt) in sham-injected rats fed corn oil (CO), but was detectable in sham-injected rats fed fish oil (FO) and more frequent still in DMH-treated rats fed corn oil (DMH-CO) and in DMH-treated rats fed fish oil (DMH-FO). Analysis of variance confirmed significant effects of DMH \( (P < 0.001) \) and fish oil \( (P < 0.001) \) at 24 h, and an independent significant effect of fish oil at 48 h \( (P < 0.001) \).

A secondary analysis was carried out to determine whether the differences in the frequency of apoptosis per crypt between groups could be attributed to systematic differences in total cell number per crypt. At 24 h the mean crypt lengths in the distal colon were: CO \((252 \pm 10 \, \mu m)\), FO \((256 \pm 11 \, \mu m)\), DMH-CO \((259 \pm 9 \, \mu m)\), DMH-FO \((274 \pm 26 \, \mu m)\) with no significant differences between groups. The data for apoptotic nuclei per crypt were normalized using crypt length as an index of cell number and re-expressed as apoptotic nuclei per unit crypt length. The distribution of differences between groups remained essentially unchanged (data not shown) and one-way analysis of variance confirmed that the DMH-FO group differed significantly from all other groups \( (P < 0.05) \).

At 48 h the crypts from the DMH-FO group \((289 \pm 19 \, \mu m)\) were significantly longer \( (P < 0.05) \) than those of the CO \((240 \pm 20 \, \mu m)\) and FO groups \((243 \pm 7 \, \mu m)\), but not the DMH-CO group \((276 \pm 24 \, \mu m)\). However, analysis of variance showed that the number of apoptotic nuclei per unit crypt length in the DMH-FO group remained significantly higher than in all other groups \( (P < 0.05) \).

The spatial distributions of apoptotic cells in crypts from the distal colons of FO, DMH-CO and DMH-FO rats at 24 and 48 h after DMH are shown in Figure 4. In both CO and FO fed rats, DMH-induced apoptosis at 24 h was maximal within the basal 10th of the crypt length (zone 1) and entirely absent in the highest three-tenths (zones 8, 9 and 10). At 48 h apoptosis in the DMH-FO group was still absent in zones 8, 9 and 10, but highest in zone 3, although this spatial trend was not statistically significant.

**Effects of DMH and lipid on crypt cell mitosis**

Crypt cell mitosis (Figure 5) was significantly lower in animals given DMH compared with controls at 24 and 48 h \( (P < 0.001) \). There were also independent effects of lipid on crypt cell mitosis at both 24 h \( (P < 0.05) \) and 48 h \( (P < 0.001) \), so that the lowest levels of all were observed in the DMH-FO group. There was an effect of site in the colon at 24 h \( (P < 0.05) \), such that mitosis was lowest in the distal colon, but this was not significant at 48 h.

**Aberrant crypt foci and crypt cell kinetics 18 weeks after exposure to DMH**

The numbers and crypt multiplicities of ACF in the treatment groups are summarized in Table I. All animals treated with DMH developed ACF in the distal colon after 18 weeks, but none were detected in the distal colons of rats given sham injections. The relative incidence of ACF was not measured in the proximal colon as previous studies have questioned the validity of using ACF as a prognostic indicator at this site \( (21) \). Rats fed the FO diet for 48 h after each DMH injection and maintained thereafter on the CO diet had approximately 50% fewer ACF per whole distal colon \( (P < 0.05) \) and per


Significance

P associated with FO at both 24 h (P 6 cm² of mucosal surface in distal colon (Fig. 5. Crypt cell mitosis in the distal and mid colon of rats, 24 and 48 h following administration of DMH, both 24 h and 48 h following administration of DMH, the present results reflect the acute cytotoxic effects of DMH after 24 h, with rapid cell proliferation. Consumption of fish oil heightens the apoptotic response in this region at both 24 and 48 h after injection, and fish oil raises levels of apoptosis and suppressed proliferation in sham-injected animals, implying that fish oil has the capacity to modulate apoptosis and proliferation even in the absence of DMH. Although cell numbers could not be determined directly, it was established that the effects of fish oil were not associated with gross changes in crypt length. Thus, the overall effect on the crypt of fish oil was to shift the balance of cell proliferation and death in a way which might be expected to reduce the tendency of mutations to become fixed in the dividing cell population.

To determine the spatial distribution of cell proliferation and death, the crypt was analysed as a uniform cylinder divided into 10 equal sections. There was relatively little variation in crypt length between groups, and the data demonstrate that the target cells for DMH-induced cytotoxicity were situated towards the base of the crypt, within regions associated with rapid cell proliferation. Consumption of fish oil heightens the apoptotic response in this region at both 24 and 48 h after administration of DMH. Potten et al. (24) have pointed out that, unlike the small intestine, induction of apoptosis by DMH in the colon occurs higher up the crypt than the probable location of the colonic stem cells, which are thought to lie in the first one or two cell positions. Using the isolated crypt technique it is difficult to derive precise cell positional data, but apoptotic cells were often seen at the very base of the crypt in all DMH-treated animals during the study, and the raised levels of apoptosis within the basal tenth of the crypt may indicate increased deletion of cells within the putative stem cell compartment.

Aberrant crypt foci are widely regarded as early markers of incipient neoplastic change in the human large intestine and in the DMH/AOM rodent model of experimental colon carcinogenesis (21,25). In the present study we employed ACF as an index of early neoplastic induction and demonstrated that dietary fish oil fed for 48 h after administration of DMH reduced the incidence of ACF in the distal colon by ~50%.

Administration of DMH leads, within 2–4 h of administration, to a wave of apoptosis coupled with suppressed proliferation, which may last for up to 5 days in the rodent colonic crypt (10,11). The data demonstrate that consumption of fish oil augments the acute cytotoxic effects of DMH after 24 h, reflected in the raised levels of apoptosis and suppressed proliferation. There was no significant interaction between lipid and treatment with DMH 48 h after injection, and fish oil raised levels of apoptosis and suppressed proliferation in sham-injected animals, implying that fish oil has the capacity to modulate apoptosis and proliferation even in the absence of DMH. Although cell numbers could not be determined directly, it was established that the effects of fish oil were not associated with gross changes in crypt length. Thus, the overall effect on the crypt of fish oil was to shift the balance of cell proliferation and death in a way which might be expected to reduce the tendency of mutations to become fixed in the dividing cell population.

Table I. Effect of diet on numbers of DMH induced aberrant crypt foci (ACF) in the distal colon

<table>
<thead>
<tr>
<th></th>
<th>ACF distal colon</th>
<th>ACF/cm² distal colon</th>
<th>1 crypt/focus (%)</th>
<th>2 crypts/focus (%)</th>
<th>3 crypts/focus (%)</th>
<th>4 crypts/focus (%)</th>
<th>5+ crypts/focus (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMH-CO</td>
<td>25.71 ± 5.19</td>
<td>0.30 ± 0.06</td>
<td>27</td>
<td>36</td>
<td>16</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>DMH-FO</td>
<td>13.28 ± 2.09</td>
<td>0.16 ± 0.02</td>
<td>18</td>
<td>44</td>
<td>18</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Significance</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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</tbody>
</table>

*Results shown are means ± SEM and percentages for seven rats per group.

No ACF were present in sham-injected animals (data not shown).

Discussion

It has been reported previously that dietary fatty acid composition can influence the initiation, promotion and progression of experimental neoplasia in the rodent large bowel in studies where lipid intake has been manipulated both before and after treatment with DMH or AOM (1–4). The present study demonstrates that consumption of n-3 PUFA in the form of fish oil, fed only for a 48 h period following administration of DMH, can significantly reduce the incidence of ACF in the distal colon of rodents killed 18 weeks later. This protective effect was associated with significantly raised levels of apoptotic cell death and suppressed proliferation within the colonic crypt, both 24 and 48 h following administration of DMH, suggesting a mechanism by which n-3 PUFA could suppress the onset of carcinogenesis in the colon. Dietary lipid intake was manipulated only after carcinogen administration in order to minimize any effects of fatty acid composition of the diet on activation and detoxification of DMH (22,23), and thus any effects this may have on the induction of carcinogenesis by DMH.

Eighteen weeks after the final DMH or sham injection, the levels of apoptosis were negligible (<0.01 apoptotic cells per crypt) in all groups. The level of mitosis was significantly higher in the distal colon of DMH-treated rats (DMH-CO; 2.40 ± 0.02, DMH-FO; 2.39 ± 0.31) compared with controls (CO; 1.93 ± 0.19, FO; 2.09 ± 0.21) after 18 weeks (P < 0.05), but there were no significant differences in the mid colon or between dietary groups.

cm² of mucosal surface in distal colon (P < 0.05), compared with rats fed CO throughout. There was no significant effect of lipid on crypt multiplicity within the ACF (Table I).

To determine the spatial distribution of cell proliferation and death, the crypt was analysed as a uniform cylinder divided into 10 equal sections. There was relatively little variation in crypt length between groups, and the data demonstrate that the target cells for DMH-induced cytotoxicity were situated towards the base of the crypt, within regions associated with rapid cell proliferation. Consumption of fish oil heightens the apoptotic response in this region at both 24 and 48 h after administration of DMH. Potten et al. (24) have pointed out that, unlike the small intestine, induction of apoptosis by DMH in the colon occurs higher up the crypt than the probable location of the colonic stem cells, which are thought to lie in the first one or two cell positions. Using the isolated crypt technique it is difficult to derive precise cell positional data, but apoptotic cells were often seen at the very base of the crypt in all DMH-treated animals during the study, and the raised levels of apoptosis within the basal tenth of the crypt may indicate increased deletion of cells within the putative stem cell compartment.
turnover during this period, fish oil serves to reduce the number of crypts harbouring mutated cells capable of participating in neoplasia.

There are several possible mechanisms which could account for the observed effects of fish oil on crypt cell apoptosis and proliferation in the colorectal mucosa following carcinogen. In the context of this study, probably the simplest and most attractive hypothesis is that of oxidative stress. Administration of DMH poses an acute oxidative stress to the colonic mucosa, although the mucosa compensates for this by raising levels of cellular glutathione and maintaining levels of glutathione reductase (26). However, DMH is reported to compromise several other antioxidant mechanisms including: (i) lowered manganese superoxide dismutase activity; (ii) reduced constitutive levels of glutathione peroxidase; and (iii) a decreased ability to induce catalase. Sensitivity to the cytotoxic effects of n-3 PUFA has been correlated to levels of antioxidant enzymes such as Se-glutathione peroxidase in the cell (27). Incorporation of highly unsaturated EPA and DHA from the fish oil diet may further compromise the antioxidant status of the cell via increased lipid peroxidation, leading to loss of cell viability or selective induction of apoptosis. Assuming the effects of dietary fish oil are due to oxidative stress we may expect it to heighten the induction of factors which regulate the response to genotoxic and cellular stress, such as p53, SAPK/JNK, p21 WAF1. We are currently investigating whether specific fatty acids can modify cell fate via these pathways following apoptotic stimuli.

A number of signalling pathways also appear to be modulated directly or indirectly by n-3 PUFA from fish oils, including the activities and levels of second messengers 1,2-diacylglycerol (DAG), ceramide, phospholipase C, the post-translational modification and membrane localization of ras-p21, and the expression of protein kinase C isozymes during tumour development in the rodent colon (7–9). Many of these are involved in regulating cell proliferation, differentiation and apoptosis, and they may be important downstream mediators of the effects seen in this study. A number of studies suggest that dietary fish oil suppresses carcinogenesis by reducing the expression of the inducible cyclooxygenase isofrom (COX-2) in the colon and thus reducing biosynthesis of prostaglandins implicated in neoplastic transformation. Increasingly high levels of COX-2 expression are generally seen with advancing stages of carcinogenesis (28) and n-3 PUFA have been reported to suppress COX-2 expression during induction of experimental carcinogenesis in the rat colon. However, it seems unlikely that induction of COX-2 was involved in the phenomena observed in the present study since its expression is not significantly induced or influenced by diet at such an early stage in the carcinogenic process (29).

In conclusion, this study demonstrates that the 48 h following administration of DMH is a critical phase in the induction of carcinogenesis by this carcinogen, and that changes in dietary lipid intake during this period have the capacity to influence the fate of crypt cells and the genesis of pre-neoplastic lesions in the rat distal colon. It seems likely that the protective effects of fish oil reported in previous experimental studies may be at least partly due to the n-3 PUFA mediated changes in apoptosis and proliferation described here. These findings may be significant for human cancer prevention, and n-3 PUFA might, in principle, be used to augment the tumoricidal effects of chemotherapeutic drugs by increasing the susceptibility of cancer cells to apoptosis (30,31). Further work is required to determine the molecular mechanisms governing the modulation of apoptosis and mitosis by n-3 PUFA in gastrointestinal epithelial tissues.

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References


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