Effect of olive oil on early and late events of colon carcinogenesis in rats: modulation of arachidonic acid metabolism and local prostaglandin E2 synthesis

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Abstract

Background—Animal model studies have shown that the colon tumour promoting effect of dietary fat depends not only on the amount but on its fatty acid composition. With respect to this, the effect of n9 fatty acids, present in olive oil, on colon carcinogenesis has been scarcely investigated.

Aims—To assess the effect of an n9 fat diet on precancer events, carcinoma development, and changes in mucosal fatty acid composition and prostaglandin (PG)E2, formation in male Sprague-Dawley rats with azoxymethane induced colon cancer.

Methods—Rats were divided into three groups to receive isocaloric diets (5% of the energy as fat) rich in n9, n3, or n6 fat, and were administered azoxymethane subcutaneously once a week for 11 weeks at a dose rate of 7.4 mg/kg body weight. Vehicle treated groups received an equal volume of normal saline. Groups of animals were colectomised at weeks 12 and 19 after the first dose of azoxymethane or saline. Mucosal fatty acids were assessed at 12 and 19 weeks. Aberrant crypt foci and the in vivo intracolonic release of PGE2 were assessed at week 12, and tumour formation at week 19.

Results—Rats on the n6 diet were found to have colonic aberrant crypt foci and adenocarcinomas more often than those consuming either the n9 or n3 diet. There were no differences between the rats on the n9 and n3 diets. On the other hand, administration of both n9 and n3 diets was associated with a decrease in mucosal arachidonate concentrations as compared with the n6 diet. Carcinogen treatment induced an appreciable increase in PGE2, formation in rats fed the n6 diet, but not in those fed the n3 and n9 diets.

Conclusions—Dietary olive oil prevented the development of aberrant crypt foci and colon carcinomas in rats, suggesting that olive oil may have chemopreventive activity against colon carcinogenesis. These effects may be partly due to modulation of arachidonic acid metabolism and local PGE2 synthesis.

Keywords: olive oil; fish oil; azoxymethane; carcinogenesis; fatty acids; prostaglandin E2, dietary fat has received considerable attention as a possible risk factor in the aetiology of colon cancer. Epidemiological studies have indicated that the amount of dietary fat is related to colon cancer incidence. Animal studies have also consistently shown a tumour promoting effect of high fat diets (20% n6 fatty acids) favour colon carcinogenesis, particularly in its post-initiation or promotional phase, whereas feeding high fat diets rich in fish oil (23.5% n3 fatty acids) decreases colon tumour incidence in both the initiation and post-initiation phase. The effect of olive oil on colon carcinogenesis has been scarcely studied. However, in one study, high fat diets containing olive oil (23.5% n9 fatty acids) have also been reported to have low colon tumour promoting effects.

Most studies evaluating the tumour promoting effect of fat in rats have used 20% (about 5% of calories) fat diets, as this fat derived energy intake would be equivalent to that of humans in Western countries. However, 4–5% of fat (about 12% of calories) is the normal recommended amount of fat for long term feeding studies in rats. Macronutrient and energy distribution of the diet is very different between humans and rats. Daily energy intake in the rat is about 1672 kJ/kg body weight, whereas in humans it is about 125–146 kJ/kg body weight. Although 20% high fat diets have been very useful for investigating the tumour promoting effect of fat in rats, the 5% level seems to be more reasonable for assessing the preventive effect of a normal content fat diet. In fact, it is not well known whether or not the effects of different fatty acid composition of diets on colon carcinogenesis persist when diets with a normal amount of fat (5% dietary fat for rats) are administered. With respect to this, it has been shown that 5% fat diets rich in eicosapentaenoic acid (EPA; n3 fatty acid) or stearic acid (saturated fatty acid) have an inhibitory effect on colon carcinogenesis as compared with 5% fat diets rich in linoleic acid (n6 fatty acid).

Abbreviations used in this paper: EPA, eicosapentaenoic acid; ACF, aberrant crypt foci; PGE2, prostaglandin E2; HPLC, high performance liquid chromatography; ARA:EPA ratio, arachidonic acid to EPA ratio.
To our knowledge, the preventive effect of normal containing 5% fat diets rich in olive oil on rat colonic carcinogenesis has not been studied. This may be relevant as oleic acid (the major fatty acid in olive oil) is the main fat component of the Mediterranean diet, and olive oil has been found to be protective against oxidative stress and carcinogenesis. A recent epidemiological study performed in France suggests that a low intake of oleic acid may increase the risk of left colon cancer. Furthermore, another recent study suggests that high consumption of monounsaturated fats, mostly derived from olive oil, would be associated with a significant decrease in the risk of colorectal cancer with wild type Ki-ras genotype. Therefore the present study was designed to examine the effect of a 5% fat olive oil based diet, in comparison with isopidic fish oil and safflower oil based diets, on the early and late phases of azoxymethane induced colon carcinogenesis. The effect of the different types of fat on both mucosal fatty acid composition and local prostaglandin E (PGE) production were also assessed.

Materials and methods

RATS AND DIETS

Four week old male Sprague-Dawley rats (about 90–100 g body weight) were purchased from B&K Universal, Barcelona, Spain. The animals were housed two to a cage in wire drop-bottom cages, to minimise coprophagia and to prevent the consumption of bedding, under controlled conditions of a 16 hour/8 hour light/dark cycle, 50% humidity, and 21°C temperature. Animals were fed ad libitum. The food cups, especially adapted for powdered diets, were replenished with fresh diet every day.

The experimental diets were prepared by Scientific Hospital Supplies (SHS) International Limited, Liverpool, UK. They were unflavoured powdered diets containing the following calculated amounts (per 100 g): fat, 5 g; protein, 20 g; carbohydrate, 64 g; fibre, 8 g; vitamins, 90 mg; minerals, 2 g; trace elements, 74 mg. The energy value for the three diets was 16.1 MJ/kg. They were made up in a defatted meal containing 5% fat diets rich in olive oil, and the n6 diet was rich in linoleic acid, the n3 diet was rich in EPA and docosahexaenoic acids, and the n9 diet was rich in oleic acid. The diets were packaged in hermetically sealed containers with nitrogen gas and stored in a refrigerator before use.

During the 15th week of the experiment (age of animals 20 weeks), food consumption was assessed in four rats selected at random from each dietary group. Each animal was housed separately. After acclimatisation for four days, the daily dietary consumption was determined over the subsequent seven days.

EXPERIMENTAL DESIGN

A total of 108 rats were randomly assigned to three dietary groups of 36 animals each (n6, n3, and n9 diets). After a one week period of adaptation, each dietary group was divided into carcinogen treated (18 rats) and vehicle treated (18 rats) subgroups. Animals intended for carcinogen treatment were given azoxymethane (Sigma Aldrich, Madrid, Spain) in saline subcutaneously once a week for 11 weeks at a dose rate of 7.4 mg/kg body weight. Animals intended for vehicle treatment were given an equal volume of normal saline subcutaneously.

At week 12, in six animals treated with azoxymethane and six controls treated with saline from each dietary group the colon was surgically removed and processed for both aberrant crypt foci (ACF) count and assay of mucosal fatty acid composition as described below. The remaining animals were colectomised 19 weeks after the first azoxymethane or saline dose, and mucosal fatty acid composition and the number, size, and location of all identifiable intestinal tumours evaluated. Only one of the animals (carcinogen treated; n3 diet) died before the scheduled termination of the experiment. Colectomy was performed through a midline laparotomy under total anaesthesia achieved with 100 mg intraperitoneal thiopental. After colectomy, animals were not allowed to recover from the anaesthesia.

In vivo local release of PGE2 was assessed in intracolonic dialysates on the day before colectomy at week 12.

ANALYSIS OF ACF

For ACF assessment, the colons were removed, opened longitudinally, and flushed with cool normal saline. Three 0.5 cm sections of the left colon, taken at 2 cm intervals, were fixed flat between filter papers in 10% buffered formalin for 24 hours. Each section was then stained with 0.2% methylene blue in saline for five minutes, rinsed in saline, and placed on microscope slides with the mucosal side up. ACFs were identified from normal crypts using a light microscope at a magnification of 40 × by their

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Fatty acid composition of experimental diets (mean of at least four samples of powdered diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid</td>
<td>n6 diet (%)</td>
</tr>
<tr>
<td>14:0</td>
<td>0.72</td>
</tr>
<tr>
<td>16:0</td>
<td>9.85</td>
</tr>
<tr>
<td>16:1n7</td>
<td>0.18</td>
</tr>
<tr>
<td>18:0</td>
<td>5.23</td>
</tr>
<tr>
<td>18:1n9</td>
<td>19.28</td>
</tr>
<tr>
<td>18:1n7</td>
<td>ND</td>
</tr>
<tr>
<td>18:2n6</td>
<td>59.12</td>
</tr>
<tr>
<td>18:3n6</td>
<td>0.33</td>
</tr>
<tr>
<td>18:3n3</td>
<td>0.50</td>
</tr>
<tr>
<td>20:4n6</td>
<td>1.09</td>
</tr>
<tr>
<td>20:5n3</td>
<td>16.91</td>
</tr>
<tr>
<td>22:0</td>
<td>0.40</td>
</tr>
<tr>
<td>22:5n6</td>
<td>ND</td>
</tr>
<tr>
<td>22:5n3</td>
<td>ND</td>
</tr>
<tr>
<td>22:6n3</td>
<td>ND</td>
</tr>
<tr>
<td>ND, not detected.</td>
<td></td>
</tr>
</tbody>
</table>
increased pericryptal zone, elliptic or circular luminal opening, and greater thickness of the epithelial lining containing one or more crypts, which were seen to stain an intense blue. Crypt multiplicity was determined as the total number of aberrant crypts per rat.

MUCOSAL FATTY ACID ANALYSIS
After colonic sections for ACF analysis had been obtained, the remaining colonic mucosa was scraped off using a microscope slide, placed in cryovials, immediately flash frozen in liquid N2, and stored at −80°C until fatty acid assay. Fatty acid assay was performed as previously described.11 Tissue samples were put in a 4:1 (v/v) methanol/benzene solution and shaken for about one minute in a vortex mixer. Afterwards they were homogenised by sonication in an ultrasound bath. Direct transesterification of fatty acids was immediately carried out by the procedure of Lepage and Roy.16 The benzene extract was evaporated under a stream of nitrogen at 40°C to complete dryness. The residue was dissolved in 100 µl benzene, and a 1 µl aliquot was injected into the chromatograph. Fatty acid methyl esters were quantified by gas liquid chromatography in a Perkin-Elmer Autosystem (Perkin-Elmer, Norwalk, Connecticut, USA) using a 30 m capillary column, 0.25 mm internal diameter, impregnated with SP 2330 as stationary phase. The fatty acid methyl esters were identified and quantified by comparison with an external standard (Sigma Chemical, St Louis, Missouri, USA). Fatty acids from C16:0 to C24:0 were measured, unidentified peaks accounting for <0.5%. They were expressed as molar percentage of total fatty acids present.

INTRACOLONIC DIALYSIS AND PGE2 ASSAY
PGE2 was assessed in vivo by intracolonic dialysis as previously described.11 Rats were anaesthetised by intraperitoneal administration of 1.5 ml/kg of a solution containing 23 mg/ml ketalar, 2 mg/ml diazepam, and 0.2 mg/ml atropine, and intracolonic dialysis was performed using hydrated Visking seamless cellulose tubing (8/32; 6.3 mm diameter; 7 cm long; Medicell International, London, UK) attached by a 10 cm polyurethane cannula to an external syringe. After the entire cannula had been inserted into the distal colon, the dialysis bag was filled with 1 ml dialysis solution, consisting of 0.3% bovine serum albumin in a solution of 120 mmol/l NaCl and 30 mmol/l KHCO3, adjusted to pH 7.9. One hour later, the fluid was withdrawn and immediately stored at −80°C. A one hour dialysis period was chosen as a compromise between the time needed for equilibration and the irritant effect of the dialysis bag on PGE2 production.10 11

The volume of the dialysate recovered at the end of the one hour period was larger than 90%. During this period, there was no evidence of active diarrhoea, faecal staining of the dialysis bag, or changes in dialysis fluid volume, suggesting that the PGE2 measurements reflected production from the adjacent tissue and were not affected by fluid production in other parts of the colon.11

Tritiated standards of PGE2 were purchased from Amerham International (Amerham, Buckinghamshire, UK). Standards of PGE2 for high performance liquid chromatography (HPLC) analysis were obtained from Sigma. All solvents used in extraction and analysis were HPLC grade. Extraction of prostaglandins was performed using SepPak C18 Plus cartridges (Waters Associates, Milford, Massachusetts, USA) by a modification of the method of Powell.20 21 In brief, after sample addition, the cartridges were successively washed with 10 ml bidistilled water (pH 3.15) and 10 ml petroleum ether. Afterwards, prostaglandins were eluted with 5 ml methyl formate. C18 cartridge recoveries were measured, after vacuum evaporation of eluates to dryness and redissolution in acetonitrile, by scintillation counting of the eluates.20 Recovery for PGE2 was 90.1 (2.7)% (mean (SD), n = 5).

PGE2 determination was carried out using reverse phase HPLC.22 The HPLC system consisted of a Perkin-Elmer HPLC Isocratic Lc 250 pump, and a variable wavelength (190–300 nm) Perkin-Elmer Lc 290 UV spectrophotometric detector. The reverse phase HPLC column used was a graph (Perkin-Elmer, Norwalk, Connecticut, USA) was used to quantify the PGE2 in the fraction.

HISTOLOGICAL ANALYSIS OF TUMOURS
Tumours larger than 1 mm in diameter which could be identified with the naked eye were excised, fixed in 10% formalin, embedded in paraffin wax, and processed individually. The specimens were cut into 5 µm thick sections, and stained with haematoxylin and eosin for histological evaluation. All the slides were coded and examined by a pathologist who was unaware of the experimental group from which the specimens had been taken. The maximum size of each tumour was measured and the tumours were classified as adenomas or adenocarcinomas. The latter were graded by the degree of differentiation (well, moderate, or poor). Tumour incidence (% animals with tumours) and tumour multiplicity (number of tumours/animal) were recorded.

STATISTICAL ANALYSIS
Results are expressed as mean (SEM) or as proportions. χ2 statistics were used to compare
qualitative variables. Significant differences between groups for quantitative parametric variables were evaluated using one way analysis of variance. Bonferroni test was used to assess where the differences occurred. Kruskall Wallis one way analysis of variance by rank and Mann Whitney U test were used for non-parametric variables. Statistical analysis was performed using SPSS for Windows 6.0 (SPSS Inc, Chicago, Illinois, USA).

Results

DIETARY INTAKE AND WEIGHT GAIN

There were no differences in the mean food intake either between azoxymethane and vehicle treated animals or among dietary groups. Figure 1 shows the weight of the animals over the course of the experiment. Rats in every dietary group gained weight at comparable rates, no matter what the treatment was (azoxymethane or saline), except for vehicle treated rats on the n3 diet, which gained significantly more weight than the other vehicle treated groups (p = 0.01 in weeks 12 and 14).

FATTY ACID ANALYSIS

Incorporation of dietary fatty acids into the colonic mucosa at week 12 was similar in saline and azoxymethane treated rats (tables 2, 3, and 4). As expected, rats fed the n9 diet had the highest mucosal concentrations of oleic acid, those fed the n3 diet had the highest concentrations of n3 long chain polyunsaturated fatty acids, and feeding the n6 diet resulted in the highest concentrations of linoleic acid in colonic mucosa.

At week 12, there were significant differences in the mucosal arachidonic acid to eicosapentaenoic acid (ARA:EPA) ratio among diets (saline and azoxymethane treated rats: p<0.0005; n6 v n9 and n3; n9 v n3). On the other hand, there were no differences in the mucosal fatty acid profile between azoxymethane and saline treated rats, except for animals fed the n6 diet. In these rats, carcinogen administration was associated with a significant

Table 2 Mucosal fatty acid profile (%) in rats fed the n9 diet at weeks 12 and 19 after the first dose of carcinogen or vehicle

<table>
<thead>
<tr>
<th>n9 diet</th>
<th>Week 12</th>
<th>Week 19</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>AOM p Value</td>
</tr>
<tr>
<td>SFAs</td>
<td>34.6 (0.45)</td>
<td>33.6 (0.24)</td>
</tr>
<tr>
<td>MUFAs</td>
<td>46.9 (1.92)</td>
<td>46.6 (0.83)</td>
</tr>
<tr>
<td>C18:1n9</td>
<td>35.1 (1.62)</td>
<td>36.03 (0.78)</td>
</tr>
<tr>
<td>n3 PUFAs</td>
<td>1.22 (0.20)</td>
<td>1.28 (0.14)</td>
</tr>
<tr>
<td>C18:3n3</td>
<td>0.15 (0.02)</td>
<td>0.16 (0.02)</td>
</tr>
<tr>
<td>C20:5n3</td>
<td>0.80 (0.09)</td>
<td>0.98 (0.11)</td>
</tr>
<tr>
<td>C22:6n3</td>
<td>4.15 (0.30)</td>
<td>4.77 (0.25)</td>
</tr>
<tr>
<td>n6 PUFAs</td>
<td>17.2 (1.88)</td>
<td>18.5 (0.74)</td>
</tr>
<tr>
<td>ARA:EPA</td>
<td>63.6 (5.5)</td>
<td>69.4 (7.44)</td>
</tr>
</tbody>
</table>

Values are expressed as mean (SEM).
*p<0.05 v controls at week 12.
†p<0.05 and ††p<0.01 v AOM at week 12.
AOM, azoxymethane; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; ARA:EPA, arachidonic acid to eicosapentaenoic acid ratio.

Table 3 Mucosal fatty acid profile (%) in rats fed the n3 diet at weeks 12 and 19 after the first dose of carcinogen or vehicle

<table>
<thead>
<tr>
<th>n3 diet</th>
<th>Week 12</th>
<th>Week 19</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>AOM p Value</td>
</tr>
<tr>
<td>SFAs</td>
<td>38.3 (1.27)</td>
<td>36.9 (1.21)</td>
</tr>
<tr>
<td>MUFAs</td>
<td>35.9 (3.10)</td>
<td>37.8 (1.43)</td>
</tr>
<tr>
<td>C18:1n9</td>
<td>23.9 (2.20)</td>
<td>25.5 (1.19)</td>
</tr>
<tr>
<td>C18:2n6</td>
<td>10.8 (2.20)</td>
<td>9.8 (1.07)</td>
</tr>
<tr>
<td>C18:3n6</td>
<td>10.12 (0.02)</td>
<td>0.13 (0.01)</td>
</tr>
<tr>
<td>C20:3n6</td>
<td>7.56 (1.27)</td>
<td>5.46 (0.74)</td>
</tr>
<tr>
<td>C20:4n6</td>
<td>3.48 (0.76)</td>
<td>2.88 (0.30)</td>
</tr>
<tr>
<td>n6 PUFAs</td>
<td>14.9 (1.81)</td>
<td>15.4 (1.51)</td>
</tr>
<tr>
<td>C22:6n6</td>
<td>7.08 (0.46)</td>
<td>7.22 (0.31)</td>
</tr>
<tr>
<td>ARA:EPA</td>
<td>6.90 (1.00)</td>
<td>5.39 (0.85)</td>
</tr>
<tr>
<td></td>
<td>1.00 (0.10)</td>
<td>0.97 (0.06)</td>
</tr>
</tbody>
</table>

Values are expressed as mean (SEM).
*p<0.05 v controls at week 12.
†p<0.05 and ††p<0.01 v AOM at week 12.
AOM, azoxymethane; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; ARA:EPA, arachidonic acid to eicosapentaenoic acid ratio.

Figure 1 shows the weight of the animals over the course of the experiment. Rats in every dietary group gained weight at comparable rates, no matter what the treatment was (azoxymethane or saline), except for vehicle treated rats on the n3 diet, which gained significantly more weight than the other vehicle treated groups (p = 0.01 in weeks 12 and 14).
increase in the molar percentage of saturated fatty acids.

At week 19, the differences in the ARA:EPA ratio between the diets were similar to those at week 12 (saline and azoxymethane treated rats: p<0.0005; n6 v n9 and n3, and n9 v n3). On the other hand, there were only slight differences in the mucosal fatty acid profile between azoxymethane and saline treated rats, with a significant decrease in the concentrations of EPA in the n3 diet group, and an increase in saturated fatty acid concentrations in animals fed the n6 diet.

Animals fed the n9 diet showed a decrease in all n6 polyunsaturated fatty acids at week 19 as compared with week 12, particularly azoxymethane treated rats. Mucosal arachidonate concentrations decreased significantly in this dietary group, reaching values similar to those of animals fed the n3 diet, and significantly lower than those in the n6 diet group (azoxymethane treated rats: p = 0.003; n6 v n9 and n3 and n9 v n3). In addition, rats fed the n9 diet showed a significant decrease in docosahexaenoic acid (C22:6n3) and a significant increase in α-linolenic acid (C18:3n3) at week 19. On the other hand, rats in the n3 diet group showed a significant decrease in oleic acid and an increase in docosahexaenoic acid concentrations at week 19 as compared with week 12, especially azoxymethane treated rats. In addition, azoxymethane treated rats in the n6 diet group showed a slight but significant increase in oleic acid concentrations and a decrease in dihomogammalinoleic acid (C20:3n6) concentrations at week 19.

ASSAY OF PGE

Figure 2 gives intracolonic PGE2 concentrations at week 12. Azoxymethane treated rats fed n6 fat released significantly higher amounts of PGE2 into the colonic lumen than both the other azoxymethane treated groups and the vehicle treated rats. In contrast, rats on the n3 diet treated with the carcinogen showed a slight but significant decrease in the release of PGE2, and rats in the n9 diet group did not show any change compared with vehicle treated rats. On the other hand, PGE2 release in vehicle treated rats showed no evidence of ACF and crypt multiplicity. Animals administered saline showed evidence of ACF formation in the

Table 4 Mucosal fatty acid profile (%) in rats fed the n6 diet at weeks 12 and 19 after the first dose of carcinogen or vehicle

<table>
<thead>
<tr>
<th>n6 diet</th>
<th>Week 12</th>
<th>Controls</th>
<th>AOM</th>
<th>p Value</th>
<th>Week 19</th>
<th>Controls</th>
<th>AOM</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFAs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUFAe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:1n6</td>
<td>19.2 (2.8)</td>
<td>18.4 (2.6)</td>
<td>0.051</td>
<td>15.8 (1.9)</td>
<td>18.4 (2.6)</td>
<td>0.051</td>
<td>15.8 (1.9)</td>
<td>18.4 (2.6)</td>
</tr>
<tr>
<td>C18:2n6</td>
<td>16.1 (0.4)</td>
<td>16.7 (0.9)</td>
<td>0.064</td>
<td>15.6 (1.0)</td>
<td>16.7 (0.9)</td>
<td>0.064</td>
<td>15.6 (1.0)</td>
<td>16.7 (0.9)</td>
</tr>
<tr>
<td>C18:3n6</td>
<td>2.42 (0.1)</td>
<td>2.84 (0.1)</td>
<td>0.087</td>
<td>1.88 (0.1)</td>
<td>2.84 (0.1)</td>
<td>0.087</td>
<td>1.88 (0.1)</td>
<td>2.84 (0.1)</td>
</tr>
<tr>
<td>ARA:EPA</td>
<td>19.8 (8.6)</td>
<td>16.1 (6.2)</td>
<td>0.029</td>
<td>15.8 (6.3)</td>
<td>16.1 (6.2)</td>
<td>0.029</td>
<td>15.8 (6.3)</td>
<td>16.1 (6.2)</td>
</tr>
</tbody>
</table>

Values are expressed as mean (SEM).
*p<0.05 and **p<0.01 v controls at week 12.
†p<0.05 and ††p<0.01 v AOM at week 12.
AOM, azoxymethane; SFAs, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; ARA/EPA, arachidonic acid to eicosapentaenoic acid ratio.
colon, whereas all azoxymethane treated rats
developed ACF. In animals fed the n9 and n3
diets, azoxymethane induced significantly less
ACF per rat than those fed the n6 diet (overall
p = 0.0062; n9 and n3 diets v n6 diet). This
was mainly due to a decrease in foci with one or
two aberrant crypts in the n9 diet group, and in
foci with four crypts in the n3 diet group.
However, the total number of aberrant crypts
per rat (crypt multiplicity) was also signifi-
cantly lower in both the n9 and n3 diet groups
than in the n6 diet group (overall p = 0.0044).

TUMOUR FORMATION

Animals treated with saline showed no evi-
dence of tumour formation at either 12 or 19
weeks. Likewise, azoxymethane treated rats
showed no evidence of tumours at week 12.
However, 19 weeks after the first injection of
azoxymethane, colonic adenocarcinomas de-
veloped in seven of 12 (58%) rats of the n9 diet
and in 10 of 12 (83%) rats of the n6 diet group
(p = 0.15). Neither benign adenomas nor
metastatic invasion of the colonic tumours to
the liver, peritoneum, or regional lymph nodes
were observed. The number of malignant
colon tumours per rat was 0.8 (0.2), 0.7
(0.3), and 2.5 (0.8) for the n9, n3, and n6 diet
groups respectively (p = 0.03; n9 and n3 v n6
diet). Tumour localisation was different in the
different dietary groups. Thus animals on the
n3 and n6 diets had a predominance of colonic
tumours in the distal half of the colon (seven of
eight tumours and 28 of 30 tumours respec-
vively) as compared with those on the n9 diet
(five of 10 tumours) (p = 0.006; n9 v n6
and n9 and n3 v n6 diet). Mean tumour size was not signi-
cantly different among the groups. However,
animals fed the n9 and n3 diets showed a lower
percentage of poorly differentiated adenocarci-
nomas than those fed the n6 diet (table 5).

Discussion

The preventive or inhibitory effect of n3 fatty
acids (fish oil) on experimental colon carcino-
genesis has been widely evaluated.19 20 Fish
oil has also been reported to suppress rectal cell
proliferation in both healthy human subjects21
and patients with colonic adenomas.22 In
contrast, the effect of n9 fatty acids on colon
carcinogenesis has been scarcely assessed.
Thus, the results of this study showing that an
experimental diet containing 5% olive oil has
an anticarcinogenic effect similar to that of n3
fatty acids may be of relevance.

The use of precancer events to predict the
effects of diets on colon carcinogenesis is of
great interest because it would simplify the
experimental design of animal studies. We
chose to investigate alterations in the occur-
rence of ACF as surrogate biomarker of carci-
nogenic changes in the rat colon during the
initiation phase of colon carcinogenesis.27–30
ACF have been recently reported to represent
preneoplastic lesions of colon cancer in both
rodents31 and humans.32 33 However, known
inhibitors of experimental colon carcinogenesis
do not always prevent ACF formation.34 This
may be related to differences in both the
experimental model and the carcinogenic regi-
men used. Thus, contrasting the effect of inter-
ventions on the premalignant biomarkers with
their late effects on colon cancer development,
as shown in this study, seems to be a prudent
approach. In the present work, n9 and n3 diets
significantly inhibited ACF formation, and
both types of diet similarly inhibited the subse-
quent development of colonic tumours when
compared with n6 fatty acids. In fact, the
effects of these diets on the number of ACF
paralleled the effects on tumours. Thus, using
the present experimental design, the effect of
dietary fatty acids on ACF numbers seems
likely to be a useful predictor of tumour occur-
cence.

It has been suggested that ACF formation
and ACF growth should be examined inde-
pendently, as an intervention could presumably
suppress the initial formation of single aberrant
crypts and/or prevent single ACF from increas-
ing in size.34 In this study, the n9 and n3 diets

\[ \text{Figure 4} \quad \text{Significant correlation between mucosal
\text{arachidonate concentrations and dialysate prostaglandin E,}
\text{(PGE2) release in carcinogen treated rats.} \]

\[ \text{Figure 5} \quad \text{(A) Comparison of total number of aberrant crypt foci (ACF) and total number
\text{of aberrant crypts (AC) per rat by dietary group (n = 6 per group); (B) comparison of the
\text{total number of ACF per number of crypts per focus and by dietary group (n = 6
\text{per group}). Results are mean (SEM) per 0.50 cm².} \]

\[ \text{Table 5} \quad \text{Histology of colon tumours in azoxymethane treated rats 19 weeks after the first
dose of carcinogen} \]

<table>
<thead>
<tr>
<th>Histological parameter</th>
<th>n9 diet (\text{(adenocarcinomas}=30))</th>
<th>n3 diet (\text{(adenocarcinomas}=8)</th>
<th>n6 diet (\text{(adenocarcinomas}=30))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well differentiated</td>
<td>3 (30)</td>
<td>2 (25)</td>
<td>6 (20)</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>7 (70)</td>
<td>4 (50)</td>
<td>6 (20)</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>0</td>
<td>2 (25)</td>
<td>18 (60)**</td>
</tr>
</tbody>
</table>

Numbers in parentheses are percentage of tumour per total colon tumours with each diet.

\[ **p = 0.01 \text{ n6 v n9 diet.} \]
decreased the number of ACF similarly. However, feeding the n9 diet was mainly associated with a reduction in ACF with one or two crypts, whereas n3 diet consumption predominantly decreased ACF with four crypts. In spite of this, there were no differences in the later development of colon carcinoma between these two groups. In agreement with these findings, the size of a focus (number of aberrant crypts in a focus) was not predictive of subsequent development of tumours in a recent study.\textsuperscript{35} The differences in ACF size in rats on the n3 and n9 diets suggest that these fatty acids act in different phases of ACF formation and growth, and thus the possibility that they may act synergistically should be explored.

Besides the incidence and multiplicity of tumours, we observed differences in the degree of differentiation of carcinomas between diets. Previous studies have produced contradictory results in relation to the effect of the n3 diet on tumour differentiation. Recently, it was shown that dietary n3 fats reduce tumour yields and improve the degree of differentiation of carcinomas at colorectal anastomosis in rats.\textsuperscript{36} These findings contrast with a previous paper showing that, although dietary EPA reduced tumour yield, the tumours were not as well differentiated as those from animals fed n6 fat.\textsuperscript{37} The present findings, showing a trend to a higher degree of differentiation with n3 diets as compared with n6 fat, are in agreement with the first study. However, the significant effect of n9 fat on the degree of differentiation has not been previously described. These results suggest an effect of n3 and n9 fatty acids on cell differentiation; the intrinsic mechanisms of such effects should be further investigated.

The antitumour effect of a fish oil diet has been attributed to its content of EPA rather than docosahexaenoic acid.\textsuperscript{38} In humans, fish oil supplementation, which is associated with a doubling of the rectal mucosal EPA content, significantly decreased the rectal mucosa cell proliferative indices.\textsuperscript{39} In addition, a stepwise reduction in tissue EPA values in the human colon adenoma dysplasia carcinoma sequence has been described.\textsuperscript{40} On the other hand, it has been suggested that the dietary n3 to n6 ratio is involved in determining the effects of fish oil on rectal cell proliferation in humans.\textsuperscript{41} In that study, fish oil supplementation in subjects eating a high fat n6 diet had no antiproliferative effect, suggesting that in these subjects n3 fat supplementation was not able to decrease the mucosal ARA:EPA ratio, in spite of increasing EPA concentrations.\textsuperscript{42} Therefore, it seems that the goal of dietary interventions in colon carcinogenesis should be to decrease the mucosal ARA:EPA ratio instead of merely increasing mucosal EPA concentrations. On the other hand, the antitumour effect of olive oil has been ascribed, in part, to its oleic acid content, the predominant fatty acid in olive oil (about 75%). The present study shows that the n9 diet significantly decreased both mucosal arachidonate concentrations and ARA:EPA ratio as compared with the n6 diet, which may in part account for the observed beneficial effect of olive oil. In addition to oleic acid, other components of olive oil, such as squalene, and flavonoid and polyphenolic compounds may have chemopreventive activity against colon carcinogenesis. With respect to this, results of a recent study showed that 1% squalene suppressed colonic ACF formation and crypt multiplicity in a model of experimental colon carcinogenesis.\textsuperscript{43} Thus the antitumour effect observed with the n9 diet in the present study may also be due to its squalene content (0.8% of the olive oil used in the n9 diet).

Oleic acid is also found in the fat of beef and poultry (30–45% of the fat) and in other vegetable oils, such as corn oil (30%), soybean oil (25%), and sunflower seed oil (33%).\textsuperscript{44} As these other fats and oils containing oleic acid generally act as promoters of chemically induced tumours in animals, it has been suggested that the oleic acid content of olive oil cannot account for its protective effect in cancer development.\textsuperscript{45} However, these other fats and oils are rich in either saturated fat or linoleic acid, and their administration is associated with an increase in the mucosal arachidonate content, and thus with an increase in the ARA:EPA ratio.

Methods for studying the role of eicosanoids in colon carcinogenesis include various in vitro assays, such as determination of the mucosal PGE\textsubscript{2} concentration in tissue homogenates,\textsuperscript{46} generation and release of PGE, by colonic biopsy specimens into the cell culture medium,\textsuperscript{21} and formation of prostaglandins from [\textsuperscript{14}C]arachidonate through mucosal cyclooxygenase activity.\textsuperscript{47} However, measurements of tissue concentrations may be misleading because eicosanoids are not stored, but produced in response to tissue trauma—for example, mucosal trauma resulting from biopsy sampling may itself activate membrane bound phospholipases.\textsuperscript{48} Similar caveats apply to tissue culture techniques.\textsuperscript{49} Over the last few years, in vivo intracolonic dialysis against rectal mucosa, as performed in our study, has been suggested to be a more reliable index of the balance between eicosanoid production and degradation than in vitro incubations of colon mucosa. In fact, it has been widely used to assess in vivo eicosanoid release in experimental and human colitis.\textsuperscript{50–52} Rectal dialysate collections probably arise from production by the adjacent colon without any contribution from secretions from other parts of the intestine.\textsuperscript{53} On the other hand, although with a dialysate time of only one hour our measurements may have underestimated PGE\textsubscript{2} concentrations, they avoided the artificial formation of PGE, induced by the irritant effect of the dialysis bag. Nevertheless, dialysate PGE\textsubscript{2}, concentrations were considerably enhanced in the carcinogen treated animals fed the n6 fat diet as compared with the n3 and n9 groups, and correlated closely with mucosal arachidonate concentrations, suggesting that they are a good estimation of the mucosal production of this prostaglandin and are not derived from bacterial microflora.

As mentioned above, one possible mechanism by which n3 and n9 fats may exert their
antitumour effect may be by influencing arachidonic acid metabolism. This fatty acid is in turn the precursor of the dienoic prostaglandins, and it has been shown that PGE2 stimulates in vitro colon cancer cell proliferation and inhibits apoptosis, suggesting a potential role for the arachidonate derived eicosanoids in human colon carcinogenesis. In this respect, previous studies have shown increased concentrations of PGE2 in colon cancer tissues in both humans and rats. It has been suggested that n3 fats may exert their anticarcinogenic effects by decreasing membrane arachidonic acid concentrations by competitive substitution, thereby reducing the synthesis of PGE2.

The present study is in agreement with this hypothesis, n3 fats suppressed the excessive production of PGE2, that accompanied ACF formation in the n6 fat diet group. On the other hand, competitive substitution of membrane arachidonate by n9 fats was also associated with unchanging intracellular PGE2, release, suggesting that the antipromotional effect of n9 fats may also be mediated through inhibition of the formation of the dienoic prostaglandins. In addition to curtailing the formation of arachidonate metabolites, it has been suggested that the antipromotional action of n3 fats may be mediated by other mechanisms such as effects on oncogene expression, 

apoptosis, and intracellular signal transduction pathways. Results of the present study are consistent with this hypothesis, as n3 fat suppressed the excessive production of PGE2 that accompanied ACF formation in the n6 fat diet group. On the other hand, competitive substitution of membrane arachidonate by n9 fats was also associated with unchanging intracellular PGE2, release, suggesting that the antipromotional effect of n9 fats may also be mediated through inhibition of the formation of the dienoic prostaglandins. In addition to curtailing the formation of arachidonate metabolites, it has been suggested that the antipromotional action of n3 fats may be mediated by other mechanisms such as effects on oncogene expression, apoptosis, and intracellular signal transduction pathways. Whether these mechanisms are also involved in the effects of n9 fats has to be further evaluated.

In conclusion, this study provides evidence that a 5% fat diet containing olive oil as compared with a 5% safflower oil diet prevents colon carcinogenesis in rats, as occurs with 5% fish oil diets. This effect may be partly due to the modulation of arachidonate metabolism and local PGE2, synthesis. Whether there is a direct inhibitory effect of olive oil and fish oil on colon carcinogenesis, or whether there is only a deleterious effect of safflower oil needs further investigation.

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