Suppressing effect of perilla oil on azoxymethane-induced foci of colonic aberrant crypts in rats

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We have investigated the modulatory effect of dietary perilla oil which is rich in the n-3 polyunsaturated fatty acid, α-linolenic acid, on the development of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) in male F344 rats. Animals were given three weekly subcutaneous injections of AOM (15 mg/kg body weight) to induce ACF. The rats were fed a basal diet containing either 12% olive oil, 12% safflower oil, 12% perilla oil, 6% perilla oil plus 6% olive oil, or 3% perilla oil plus 9% olive oil for 5 weeks, starting 1 week before the first dosing of AOM. All rats were sacrificed 2 weeks after the last AOM injection. The amount of food consumed and body weight gain were identical among every dietary group. The frequency of ACF was significantly lower in the rats fed 12% perilla oil than in those fed 12% olive oil or 12% safflower oil (P < 0.01 and P < 0.05, respectively). The suppressive effect of perilla oil was dose-dependent, as the number of ACF was 20.7, 40.7 and 47.4% of those of the 12% olive oil-fed controls in rats fed 12% perilla oil, 6% perilla oil plus 6% olive oil, or 3% perilla oil plus 9% olive oil, respectively. Perilla oil significantly reduced ras expression as well as the AgNORs count (cell proliferation biomarkers) in the colonic mucosa, as compared with olive oil or safflower oil (P < 0.01, respectively). Marked increases in n-3 polyunsaturated fatty acids in membrane phospholipid fractions and decreased PGE2 levels were observed in colonic mucosa of perilla oil-fed rats. These results suggest that perilla oil, even in small amounts, suppresses the development of aberrant crypt foci, and is therefore a possible preventive agent in the early stage of colon carcinogenesis.

Introduction

Epidemiologic studies have shown that dietary factors play a key role in the etiology of colorectal cancers (1–3). Western-style diets, rich in animal fat and poor in fiber, have been suggested to associate with an increased risk of colon cancer (4). On the other hand, studies in Alaskan and Greenland Eskimos have suggested that their low incidence of colon cancer is linked to their high dietary consumptions of fish oil products (5,6). Thus, the types or composition of fatty acids in oil may be significant determining factors in colon carcinogenesis.

Fish oil contains an appreciable amount of n-3 polyunsaturated fatty acids (PUFAs*), namely eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) and a small amount of the n-6 PUFA, linoleic acid (C18:2 n-6). Perilla oil is also rich in vegetable n-3 PUFA, α-linolenic acid (C18:3 n-3). α-Linolenic acid is converted to EPA or DHA after enzymatic desaturation and elongation in the liver. Attention has been paid to n-3 PUFAs in the prevention of colon carcinogenesis. Studies in animal models have shown that such oils rich in n-3 PUFAs suppress chemically induced colon cancer in rats (7–12). n-3 PUFAs have also been reported to suppress rectal cell proliferation in humans (13,14). These reports have shown that colon tumorigenesis was suppressed when n-3 PUFA was administrated in both the initiation and post-initiation phase (10,11) or in the post-initiation phase only (12). However, limited information is currently available on the effect of perilla oil in the initiation phase of colon carcinogenesis.

Aberrant crypt foci (ACF) have been recently reported to be putative neoplastic lesions of colon cancer in both rodents (15) and humans (16), and ACF with large numbers of crypts (four or more) per focus have been proposed as intermediate biomarkers for colon carcinogenesis (17-19). ACF have been shown to be associated with c-fos (20) and ras mutations in rats (21,22) and humans (23). They have been demonstrated to be useful biomarkers for screening chemopreventive agents against colon cancer (18). Recently, DHA has been reported to suppress the formation and growth of ACF, as well as colon tumors, in rats (19).

Here, we report an inhibitory effect of perilla oil on the development of ACF induced by azoxymethane (AOM) in rats. The aim of the present study was to examine whether perilla oil is effective in the initiation phase of colon carcinogenesis and to gain further insight into its mechanism. The effect was dose-dependent, and was accompanied by a reduction in colonic cell proliferation as revealed by the silver-stained nucleolar organizer region protein (AgNORs) count as well as ras expression. A rapid replacement of fatty acids in the mucosal phospholipid fraction by n-3 PUFAs and a suppression of prostaglandin E2 (PGE2) synthesis in the colonic mucosa of perilla oil-fed rats was also found, suggesting one of the mechanisms of perilla oil’s antitumor effect.

Materials and methods

Animals

Four-week-old male F344 rats (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were quarantined for 1 week, and randomized into experimental and control groups. Animals were housed, two to three rats each, in a plastic cage in a holding room under constant conditions of 22 ± 2°C, 50 ± 10% humidity, and a 12-h-light-dark cycle. They had free access to drinking water and food.

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Chemicals and dietary fats
AOM was purchased from Sigma Chemical Co., St Louis, MO. The dietary fats, olive oil, safflower oil and perilla oil were supplied by the Ajinomoto Co., Tokyo, Japan. The fatty acid composition of the oils was as follows: olive oil—9.6% palmitic acid (C16:0), 2.7% stearic acid (C18:0), 80.6% oleic acid (C18:1); safflower oil—6.2% palmitic acid, 1.6% stearic acid, 11.2% oleic acid, 80.5% linoleic acid and 0.4% α-linolenic acid; and perilla oil—6.2% palmitic acid, 2.0% stearic acid, 17.7% oleic acid, 15.2% linoleic acid and 56.0% α-linolenic acid.

Diets
The experimental diets were prepared once a week by adding olive oil, safflower oil or perilla oil to the basal laboratory diet (Oriental Yeast Co., Tokyo, Japan). The final w/w composition of the diet was 20% casein, 59% sucrose, 4% cellulose, 0.15% choline chloride, 4% mineral mixture, 1% vitamin mixture and 12% test oil. The diet had standard levels of nutrients.

Identification of ACF
The colon was divided into three equal portions, labeled the upper, middle and lower colon, and fixed in 10% buffered formalin solution. All portions of the colon were embedded in paraffin, and two serial sections (3 μm thick) were made. One section was used for staining AgNORs, and the other was stained with hematoxylin and eosin for histologic examination. AgNORs staining was carried out as described previously (27). The AgNORs count of the mucosal epithelium of the colon was determined in all epithelial cells lining the 20 most-oriented crypts, in which the base, lumen and top of the crypt could be observed completely. The AgNORs-stained nuclei were counted at a magnification of ×400. Data are expressed as number of AgNORs/nucleus.

Results
The mean body weight and food intake of the rats receiving AOM were lower than those of rats without AOM treatment in each dietary group (Table I). However, no significant difference was observed in either the mean body weights or the mean food intakes of the AOM-treated rats in the different dietary groups. No neoplasm was found on macroscopic or microscopic examination of the organs of any rat in each group.

The rats treated with AOM (groups 1–5) showed a 100% incidence of ACF (Table II). No ACF developed in the colon of rats without AOM treatment (groups 6–10). Both the number of ACF/cOLON and the number of aberrant crypts/colon were significantly decreased in the S12 group as compared with the O12 group (P < 0.01, respectively) (Table II). The number of ACF/colon and the number of aberrant crypts/colon were also significantly reduced in the P12 group as compared with the O12 (P < 0.01, respectively) and S12 group (P < 0.05, respectively) (Table II). The number of aberrant crypts/focus in the P12 and S12 groups were significantly smaller than that in the O12 group (P < 0.05). The suppressive effect of perilla oil on the development of ACF was obvious in both small- to large-sized foci, as the number of foci consisting of one to eight crypts was equally decreased by perilla oil administration, as compared with safflower oil (P < 0.05) or olive oil feeding (P < 0.01) (Figure 2). The suppressive effect of perilla oil on the number of ACF/colon and on the total number of aberrant crypts/colon was dose-dependent (Table II). Both numbers were significantly lower in the P309 group than in the O12

\[
\begin{array}{|c|c|}
\hline
\text{Group no.} & \text{dietary fats} \\
\hline
1 & \text{test oil} \\
2 & \text{test oil} \\
\hline
\end{array}
\]

10 rats each
Table I. Mean body weight and mean diet intakes of rats in each test group

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Diet</th>
<th>With AOM treatment</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean body weight (g)</td>
<td>Mean food intake (g/rat/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>229 ± 10</td>
<td>12.9 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>224 ± 4</td>
<td>11.0 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>227 ± 12</td>
<td>12.5 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>233 ± 13</td>
<td>13.3 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>223 ± 14</td>
<td>13.6 ± 2.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Diet</th>
<th>Without AOM treatment</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean body weight (g)</td>
<td>Mean food intake (g/rat/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>250 ± 10</td>
<td>12.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>245 ± 12</td>
<td>13.4 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>249 ± 9</td>
<td>13.0 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>238 ± 5.0</td>
<td>12.9 ± 5.0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>243 ± 12</td>
<td>12.1 ± 14</td>
<td></td>
</tr>
</tbody>
</table>

Rats were fed with a diet containing 12% olive oil (O12), 12% Safflower oil (S12), 12% perilla oil (P12), 3% perilla oil plus 9% olive oil (P3O9), or 6% perilla oil plus 6% olive oil (P6O6).

Values represent mean ± SD, at the termination of the study (n = 10).

Table II. Effect of test oils on AOM-induced ACFs in rat colon

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>Incidence</th>
<th>No. of ACF/colon</th>
<th>No. of aberrant crypts/colon</th>
<th>No. of aberrant crypts/focus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AOM + O12</td>
<td>5/5</td>
<td>155.0 ± 10.8</td>
<td>260.4 ± 16.2</td>
<td>1.68 ± 0.08</td>
</tr>
<tr>
<td>2</td>
<td>AOM + S12</td>
<td>5/5</td>
<td>81.8 ± 8.6b</td>
<td>119.6 ± 11.8b</td>
<td>1.46 ± 0.04b</td>
</tr>
<tr>
<td>3</td>
<td>AOM + P12</td>
<td>5/5</td>
<td>40.0 ± 14.8bc</td>
<td>50.3 ± 3.1bc</td>
<td>1.54 ± 0.09bc</td>
</tr>
<tr>
<td>4</td>
<td>AOM + P6O6</td>
<td>5/5</td>
<td>78.5 ± 11.3b</td>
<td>126.5 ± 21.0b</td>
<td>1.61 ± 0.08</td>
</tr>
<tr>
<td>5</td>
<td>AOM + P3O9</td>
<td>5/5</td>
<td>91.4 ± 13.8b</td>
<td>148.6 ± 26.3b</td>
<td>1.62 ± 0.07</td>
</tr>
</tbody>
</table>

For definitions of dietary groups, see the note to Table I.

aNumbers of rat colon with ACF/total numbers of colon are scored
bSignificantly different from the AOM + O12 group; P < 0.01.
cSignificantly different from the AOM + S12 group; P < 0.05.
Values are mean ± SD (n = 5).

Fig. 2. Size distribution of AOM-induced ACF in the colon of O12, S12 and P12 groups. Columns, mean; bars, SD (n = 5). (a) Significantly different from the O12 group; P < 0.01. (b) Significantly different from the S12 group; P < 0.05.

The fatty acid compositions of the colonic mucosa in the O12, S12 and P12 groups with AOM treatment are shown in Table III. The data were summarized by grouping the fatty acids into saturated fatty acids (SFAs), MUFAs, n-6 PUFAs and n-3 PUFAs. MUFAs and n-6 PUFAs were the major components in the O12 group and in the S12 group, respectively, corresponding to the composition of the dietary fatty acids. Only trace amounts of n-3 PUFAs were detected in the P12 group, indicating that as little as 3% perilla oil inhibited the development of ACF. The suppression was also obvious in ACF of all the sizes (Figure 3).

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For definitions of dietary groups, see the note to Table I.

aNumbers of rat colon with ACF/total numbers of colon are scored
bSignificantly different from the AOM + O12 group; P < 0.01.
cSignificantly different from the AOM + S12 group; P < 0.05.
Values are mean ± SD (n = 5).

O12 and S12 groups. In the P12 group, n-3 PUFAs increased markedly. EPA and DHA were the major n-3 PUFAs components. The mucosal n-3 PUFAs/MUFAs ratio was 0.98 in the P12 group and 0.04 in the O12 group. The n-3 PUFAs/n-6 PUFAs ratio was 1.10 in the P12 group and 0.03 in the S12 group.

A high PGE2 level in the colon was observed in the S12 group (Figure 4). The PGE2 level in the P12 group was significantly (P < 0.05) decreased, to approximately half that of the S12 group. The PGE2 level of the O12 group was...
Table III. Fatty acid composition of the phospholipid fractions of the colonic mucosa of AOM-treated rats in the O12, S12 and P12 groups

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>SFAs</th>
<th>MUFA</th>
<th>n-6 PUFAs</th>
<th>n-3 PUFAs</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C16:0</td>
<td>C18:0</td>
<td>C16:1</td>
<td>C18:1</td>
<td>C18:2</td>
</tr>
<tr>
<td>1</td>
<td>AOM + O12</td>
<td>27.7 ± 2.5</td>
<td>11.3 ± 2.5</td>
<td>4.0 ± 1.1</td>
<td>32.2 ± 4.1*</td>
<td>6.0 ± 0.7</td>
</tr>
<tr>
<td>2</td>
<td>AOM + S12</td>
<td>17.2 ± 2.3</td>
<td>6.7 ± 0.4</td>
<td>2.9 ± 0.6</td>
<td>14.4 ± 1.0a</td>
<td>43.5 ± 3.0a</td>
</tr>
<tr>
<td>3</td>
<td>AOM + P12</td>
<td>18.2 ± 1.6</td>
<td>10.3 ± 2.0</td>
<td>2.7 ± 0.6</td>
<td>20.5 ± 1.8</td>
<td>16.6 ± 1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28.5 ± 3.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For definitions of dietary groups, see the note to Table I.

*Significantly different from the other two groups; \( P < 0.05 \).

Values represent per cent of total fatty acids in each oil (mean ± SD, \( n = 5 \)).

Fig. 4. PGE2 levels in the colonic mucosa of AOM-treated rats in the O12, S12 and P12 groups. Columns, mean; bars, SD (\( n = 5 \)). (a) Significantly different from the O12 and S12 groups; \( P < 0.05 \).

Table IV. Number of cells/crypt column and AgNORs counts of colonic mucosa in each group

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>No. of cells/ crypt column</th>
<th>AgNORs count/nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AOM + O12</td>
<td>42.4 ± 3.9</td>
<td>1.60 ± 0.14</td>
</tr>
<tr>
<td>2</td>
<td>AOM + S12</td>
<td>32.5 ± 2.0a</td>
<td>1.51 ± 0.17</td>
</tr>
<tr>
<td>3</td>
<td>AOM + P12</td>
<td>33.4 ± 2.9b</td>
<td>1.35 ± 0.21b</td>
</tr>
<tr>
<td>4</td>
<td>AOM + P3O6</td>
<td>34.3 ± 3.2a</td>
<td>1.41 ± 0.12b</td>
</tr>
<tr>
<td>5</td>
<td>AOM + P3O9</td>
<td>35.3 ± 2.9a</td>
<td>1.54 ± 0.16</td>
</tr>
</tbody>
</table>

For definitions of dietary groups, see the note to Table I.

*Significantly different from the AOM + O12 group; \( P < 0.01 \).

Values are mean ± SD (\( n = 5 \)).

approximately the same as that of the S12 group, although the occurrence of ACF was much higher in the O12 group.
The mean number of cells/crypt column was significantly smaller (\( P < 0.01 \)) in the S12 and the P12 groups than in the O12 group (Table IV). The mean AgNORs count/nucleus in the P12 group was significantly lower (\( P < 0.01 \)) than those in the O12 and S12 groups. Both numbers were suppressed in a dose-dependent fashion by increasing the amount of perilla oil (Table IV).

The expression of c-H-ras mRNA was reduced in the P12 group as compared to those in the S12 and O12 groups (Figure 5). The expression in the S12 group was slightly reduced as compared to those in the O12 group.

Discussion

In the present study, perilla oil clearly suppressed the development of AOM-induced ACF in rat colons in a dose-dependent fashion, suggesting that dietary n-3 PUFA was effective in the initiation phase of colon carcinogenesis. Treatment with perilla oil also reduced the AgNORs number and the height of the colonic mucosa. After 6 weeks of perilla oil treatment, the fatty acid compositions of mucosal membrane phospholipids were completely changed, and there were significantly reduced PGE2 levels in the colonic mucosa. It is noteworthy that a 3% perilla oil diet reduced the number of ACF to less than half that of 12% olive oil controls. These results are consistent with the report by Narisawa et al. (11) in which a 3% perilla oil feeding significantly suppressed the incidence of colon tumors, as compared with 12% safflower oil. In the current study, there was no significant difference in food consumption or body weight gain among the dietary groups. Therefore, the suppressing effect of perilla oil on occurrence of ACF was not related to calorie intake.

Unexpectedly, the development of ACF was highest in the
olive oil group, and the results were reproducible in three independent experiments (data not shown). Epidemiologic studies have indicated a low mortality from colon cancer in the areas where a large amount of olive oil is consumed (30,31). However, the effect of oleic acid is not so obvious in animal experiments, as a previous study reported a similar incidence of colon tumors in rats fed diets supplemented either with palm oil, rich in oleic acid (40% of total fatty acids) or with safflower oil (10). Moreover, the results in the present study were obtained in the initiation phase, therefore the tumor promoting effect of safflower oil might not be exerted in such an early phase of carcinogenesis. In fact, the development of aberrant crypt foci has also been reported to be enhanced by the supplementation with olive oil as compared to the supplementation with corn oil which is rich in n-6 PUFAs (32). One possible mechanism is that olive oil may positively influence carcinogen activation in the liver (33). Further studies will be needed to clarify the effect of oleic acid and linoleic acid on the development of colonic ACF.

Although the mechanism(s) of the tumor promoting effect of animal fat and of the lack of such an effect by fish or perilla oil is not fully understood, some explanations have been proposed. One is that high dietary fat increases the secretion of secondary bile acids, which are known to act as colon tumor promoters (34,35). However, a detailed experiment by Narisawa et al. (10) has shown that the amount and concentration of total and secondary bile acids in the feces are not different between rats fed perilla oil and those fed safflower oil. Therefore, it seems unlikely that the inhibitory effect of perilla oil can be attributed to a change in fecal bile acids. Another possible explanation is altered PG synthesis. PGs, particularly the type-2 series, are believed to be closely involved in colon carcinogenesis, as increased PG levels have been found in colon cancer tissues (36). PGE2 induces hyperproliferation in colonic mucosa (37), and inhibitors of PG synthesis, such as indomethacin, inhibit colon carcinogenesis in rats (38-40). n-3 PUFAs has been reported to inhibit the production of the type-2 series of eicosanoids, including PGE2, from arachidonic acid (41-43). In the present study, the concentration of PGE2 in the colonic mucosa was suppressed by perilla oil. However, the PGE2 concentration did not correlate with the development of ACF in the olive oil group. Since the effect of PGE2 has not been reported to exerted in the promotion phase (44), it might not be the case in the initiation phase. These results led us to consider another mechanism by which perilla oil exerts its inhibitory effect on the development of ACF. In the present study, perilla oil significantly reduced AgNORs count/nucleus, suggesting that perilla oil decreased the number of cells in S-phase, and thus protected DNA from injury by AOM. The observation that perilla oil suppressed c-H-ras expression, another cell proliferation biomarker, also supports the possibility that perilla oil exerted an antiproliferative effect on the mucosal cells and thus protected them from the carcinogenic stimuli by AOM. Marked alterations in fatty acid composition were also observed in the colonic mucosal membranes of rats fed perilla oil. Oral supplementation with n-3 PUFAs is known to induce a selective incorporation of n-3 PUFAs and a competitive exclusion of n-6 PUFAs in the membrane phospholipid fractions (45). As changes in the ratio of n-3 to n-6 PUFAs in the membrane could affect the function of the membrane itself and/or membrane bound receptors, such as epidermal growth factor receptor (EGFR) (46), it may be possible that such change in membrane compositions altered the sensitivity of the cells to growth and carcinogenic stimuli (47). Another possible mechanism may be antioxidative action which is exerted by micronutrients, such as vitamin E, in the oils. The concentrations of vitamin E were 465 μM in olive oil, 1023 μM in safflower oil and 1493 μM in perilla oil, which we measured using high performance liquid chromatography (Onogi et al., unpublished observations). Therefore, the development of ACF may be related inversely to the amount of dietary intake of vitamin E, as has been suggested very recently (48). Further studies are currently under investigation in our laboratory to elucidate the mechanism of perilla oil's effect.

Our data that a vegetable n-3 PUFA, α-linolenic acid, effectively inhibited the development of colonic ACF, an early biomarker of colon carcinogenesis, support previous reports in which supplementation with perilla oil inhibited colon tumorigenesis in rats (10-12). Moreover, even a small amount of perilla oil (3%) could exert a significant suppressing effect. These findings support the possibility for clinical use. In addition, α-linolenic acid in perilla oil is much more chemically stable than EPA and DHA in fish oil (49), suggesting that the use of perilla oil seems advantageous for the long-term clinical applications. In fact, a clinical trial in which perilla oil is being used for the treatment of inflammatory bowel diseases, including Crohn's disease, has already started in Japan. We are now preparing for a clinical trial for an interventional study to prevent colon cancer with the use of perilla oil.

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References


