Synergistic suppression of azoxymethane-induced foci of colonic aberrant crypts by the combination of β-carotene and perilla oil in rats

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The modulating effect of the combined dietary feeding of β-carotene and perilla oil, which is rich in α-linolenic acid, on the development of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) was investigated in male F344 rats. Rats received oral administration of β-carotene (0, 50 or 200 mg/kg body weight/day) and fed a basal diet containing either 12% olive oil, 3% perilla oil plus 9% olive oil, or 12% perilla oil. A dose-dependent suppressive effect of perilla oil was found. The numbers of ACF were 42.0 and 18.4% of those of the 12% olive oil-fed controls in the rats fed 3% perilla oil plus 9% olive oil and 12% perilla oil, respectively. The development of ACF was also reduced significantly by the addition of dietary β-carotene in each of the oil-fed groups (P < 0.05, respectively). The suppression by the combination of β-carotene and perilla oil was synergistic, as the numbers of ACF were 12.9 and 8.9% of those of the 12% olive oil-fed controls in β-carotene-treated rats fed 3% perilla oil plus 9% olive oil and 12% perilla oil, respectively. β-Carotene plus perilla oil also suppressed the numbers of silver-stained nucleolar organizer regions and the expression of ras mRNA in the colonic mucosa (cell proliferation biomarkers). Following administration of β-carotene, a significant increase in the concentration of intact β-carotene molecules was found in the colonic mucosa, livers, and sera. However, no accumulation of retinoids was observed in the colonic mucosa, suggesting that the inhibitory effect may not be related to the provitamin A activity. These results suggest that the combination of β-carotene and perilla oil may be useful in the prevention of colon cancer.

Introduction

One possible means to prevent colon cancer is a dietary supplementation with agents that have inhibitory effects on the carcinogenesis. n-3 Polyunsaturated fatty acids (PUFAs*), namely eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), are the potential candidates for such supplements. Epidemiological investigations have suggested that fish oil, rich in n-3 PUFAs, is associated with a low incidence of colon cancer in Alaskan and Greenland Eskimos (1,2). Studies using animals also have demonstrated that a high fish oil diet significantly reduces the incidence of chemically-induced colon cancer, as compared with diets high in animal or vegetable oils (3–5). Fish oil also has been reported to suppress rectal cell proliferation in both normal human subjects (6) and patients with colonic adenomas (7). Narisawa et al. (8,9) and Hirose et al. (10) have reported that perilla oil, a vegetable oil rich in a n-3 PUFA, α-linolenic acid (C18:3 n-3), also suppresses colon cancer in rats. α-Linolenic acid is converted to EPA or DHA following enzymatic desaturation and elongation in the liver. We have previously reported the suppressive effect of perilla oil on the development of colonic aberrant crypt foci (ACF), suggesting that n-3 PUFA exerts its antitumorigenic action when fed during the initiation phase of colon carcinogenesis (11).

Another possible dietary agent against colon carcinogenesis is β-carotene. A recent large trial in China has shown that a dietary supplementation with a combination of β-carotene, α-tocopherol and selenium reduced the mortality due to oesophageal and gastric cancers (12). In contrast, another clinical trial in Finland, using a supplementation with β-carotene or α-tocopherol, failed to reduce the incidence of lung cancer among male smokers (13). However, β-carotene is still considered to be a potent preventative agent. In fact, several reports have shown its preventative effect on colon carcinogenesis in animal experiments (14–16).

We tried to determine whether n-3 PUFAs and β-carotene could work synergistically in the prevention of colon tumorigenesis by assessing the development of ACF. ACF have been recently reported to represent preneoplastic lesions of colon cancer in both rodents (17,18) and humans (19), and it has been proposed that their formation serves as an intermediate biomarker for colon carcinogenesis (20–22). The development of ACF also has been associated with c-fos (23) and ras expression in rats (24) and humans (25), as well as with ras mutations in rats (26,27). Recently, DHA, as well as β-carotene, has been reported to suppress the formation and growth of ACF in rats (15,16,22). The aim of the present study is to test whether the combination of both agents has an additive or synergistic effect on the development of ACF during the initiation phase of colon carcinogenesis.

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 or 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.

Chemicals and dietary fats

Azoxymethane (AOM) was purchased from Sigma Chemical Co., St Louis, MO. β-Carotene was obtained from Roche Japan Inc., Tokyo, Japan. Olive oil and perilla oil were supplied by the Ajinomoto Co., Tokyo, Japan. The fatty acid composition of the olive oil was 9.6% palmitic acid (C16:0), 2.7% stearic acid (C18:0), 80.6% oleic acid (C18:1), 5.8% linoleic acid (C18:2 n-6) and 0.6% α-linolenic acid (C18:3 n-3). The composition of perilla oil was 6.2% palmitic acid, 2.0% stearic acid, 17.7% oleic acid, 15.2% linoleic acid and 56.0% α-linolenic acid.
Diet

The experimental diets were prepared once a week by adding either olive oil or perilla oil to the basal laboratory diet (Oriental Yeast Co., Tokyo, Japan). The final weight/weight composition of the semi-purified diets 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. The final composition of the test oil was 12% olive oil in the \(O_1\) diet, 12% perilla oil in the \(P_1\) diet and 3% perilla oil plus 9% olive oil in the \(P_3O_9\) diet. The ratios of mono-unsaturated fatty acid (MUFA): n-6 PUFAs: n-3 PUFAs were 1:0.07:0.01 in the \(O_2\) diet and 1:0.74:2.7 in the \(P_1\) diet. The food was sealed in air-tight plastic bags under nitrogen gas and stored at \(-20^\circ\)C until use. The food in the animal cage was shaded from light and changed every other day. \(\beta\)-Carotene was administered orally at a dose of 0, 50 or 200 mg/kg body weight/day in distilled water.

Experimental design

A total of 140 rats were divided into 14 groups of 10 rats each (Figure 1). The 6-week-old rats in groups 1–7 were given subcutaneous injections of AOM (15 mg/kg body weight) once a week for 3 weeks. The rats in groups 8–14 served as AOM-negative controls. The rats in groups 1 and 8 were fed the \(O_1\) diet. Groups 2 and 9 were fed the \(O_2\) diet plus 50 mg/kg/day of \(\beta\)-carotene, groups 3 and 10 the \(O_2\) diet plus 200 mg/kg/day of \(\beta\)-carotene, groups 4 and 11 the \(P_1\) diet alone, groups 5 and 12 the \(P_1\) diet plus 50 mg/kg/day of \(\beta\)-carotene, groups 6 and 13 the \(P_1\) diet alone, and groups 7 and 14 the \(P_1\) diet plus 50 mg/kg/day of \(\beta\)-carotene. Each group received assigned diet starting at 5 weeks of age. The daily intake of the diets was recorded. All rats were provided with the diet and tap water ad libitum, and were weighed weekly.

All animals were killed at 4 weeks following the first administration of AOM, and complete necropsies were performed. The colons of five rats in each group were used to determine the presence of ACF and silver-stained nuclear organizer proteins (AgNORs). The colons of the remaining five rats were used to determine the \(\beta\)-carotene and retinoid concentrations, and mRNA expression of \(c-H-ras\) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Identification of ACF

At the termination of the study period, the colons of five animals in each group were flushed with saline, opened longitudinally from caecum to anus, placed between two pieces of filter paper and fixed in buffered 10% formalin solution for 24 h. The colons were stained with 0.2% methylene blue in saline according to the method of Bird (17). The number of ACF/colon and the number of aberrant crypts in each focus were counted microscopically at a magnification of ×40. The criteria used to identify an aberrant crypt focus topographically (17,20,21) were as follows: (1) increased size; (2) thicker epithelial cell lining; and (3) increased pericryptal zone relative to normal crypts.

AgNOR determination

The colon was divided into three equal portions, labelled proximal, middle and distal colon, and fixed in 10% buffered formalin. All portions of the colon were embedded in paraffin and two serial sections (3 µm thick) were prepared. One section was used for staining for AgNORs, and the other was stained with haematoxylin and eosin for histological examination. AgNOR staining was performed as described previously (28). The AgNOR count of the mucosal epithelium of the colon was determined at a magnification of ×400 in 20 well-orientated crypts in which the base, lumen and top of the crypt could be observed completely. Data are expressed as the number of AgNORs/crypt.

Quantitation of \(\beta\)-carotene and retinoids

Concentrations of \(\beta\)-carotene, retinoil and retinyl esters were determined in the serum, liver and colonic mucosa. Extraction and quantitation of \(\beta\)-carotene was performed according to the method of Schnitz et al. (29). Concentrations of retinoids were determined as described previously (30).

Northern blot

RNA was extracted according to the method of Chomczynski and Sacchi (31). Northern hybridization was performed as described previously (32), utilizing a \(^{32}\)P-labelled probe specific either for human \(c-H-ras\) (Japanese Cancer Research Resources Bank) or GAPDH.

Statistical analysis

The significance of the differences between group means was analysed by Dunnett's t-test.

Results and discussion

The mean body weight and food intake of the rats receiving AOM alone were slightly lower than those of the rats not receiving AOM (224–233 g for the AOM-treated rats and 238–250 g for the rats not receiving at week 5). The mean

food intake of the AOM-treated rats ranged between 11.0–13.6 g/rat/day and those of the rats not receiving AOM ranged between 12.1–13.4 g/rat/day. However, no significant differences were observed in the mean body weight or the mean food intake among the AOM-treated rats in the different dietary groups. Therefore, the suppressive effect of perilla oil and β-carotene on ACF was unrelated to caloric intake. No neoplasms were found in all animals in each group.

The rats treated with AOM (groups 1–7) had a 100% incidence of ACF (Table I). No ACF developed in colons of rats not treated with AOM (groups 8–14). Both the numbers of ACF/colon and the numbers of aberrant crypts/colon were decreased significantly in the β-carotene-treated rats in each dietary group (Table I). The number of aberrant crypts/focus in the β-carotene-treated rats were also less than in the control rats in the P3O9 group (P < 0.05). Therefore, β-carotene clearly suppressed the development of ACF in rat colons irrespective of dietary oil supplementation. Similar suppressive effects were observed in the groups receiving 50 and 200 mg/kg body weight/day of β-carotene and the O12 diet, suggesting that 50 mg/kg body weight/day of β-carotene is sufficient for the antitumorigenic effect. In fact, previous studies reported that a small amount (0.9 mg/10 g diet) of β-carotene suppressed significantly the development of ACF in rat colon (15,16).

The numbers of ACF/colon and aberrant crypts/colon were also reduced significantly in the P3O9 and the P12 groups as compared to the O12 group, irrespective of β-carotene-treatment (P < 0.01). The numbers of ACF/colon were 42.0 and 18.4% of those of the 12% olive oil-fed rats in the rats fed 3% perilla oil plus 9% olive oil and 12% perilla oil, respectively. These findings confirm our previous report that a small dose of perilla oil suppressed significantly the development of ACF (11). An important observation in the present study is that β-carotene and perilla oil showed a synergistic suppressive effect as shown in Table I. It is noteworthy that a 3% perilla oil diet plus β-carotene had a similar effect as 12% perilla oil plus β-carotene. Thus, the effect of even a small amount of perilla oil in combination with β-carotene may be clinically useful. The suppression was observed in ACF of all the sizes, as the number of both small and large foci (with up to eight crypts) were reduced by the combination of perilla oil and β-carotene (Figure 2).

The mean AgNOR count/nucleus values were significantly less in the β-carotene-treated rats than in those not receiving β-carotene in the O12 group (Table II). The expression of ras mRNA in colonic mucosa was also reduced by the administration of perilla oil plus β-carotene (Figure 3). These results suggest that the supplementation with perilla oil plus β-carotene decreases the number of cells in S-phase, and thus protects DNA from the injury by AOM.

Although the mechanism by which β-carotene suppresses the development of ACF is still to be elucidated, some possibilities have been proposed. β-Carotene may exert antitumorigenic activity independent of its provitamin A activity (33). Our previous work employing a mouse skin papilloma model also suggested that intact β-carotene is antitumorigenic without being converted to retinoids (34). In the present study, significant concentrations of β-carotene were found not only in the sera and livers, but also in the colonic mucosa following dietary supplementation with β-carotene (Table III). A dose-dependent increase in β-carotene concentrations was observed in the tissue (4.60 nmol/g colonic mucosa at the dose

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**Fig. 2. Size distribution of AOM-induced colonic ACF of treated rats.** Columns, means; bars, SD (n = 5). (a) Significantly different from the O12 group; P < 0.01. (b) Significantly different from the O12 group; P < 0.05. (c) Significantly different from the O12+BC50 group; P < 0.01. (d) Significantly different from the P3O9 group; P < 0.01. (e) Significantly different from the P3O9 group; P < 0.05. (f) Significantly different from the P12 group; P < 0.01. (g) Significantly different from the P12 group; P < 0.05.

**Table II. Number of cells/crypt column and AgNORs/nucleus in the colonic mucosa of AOM-treated rats**

<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>37.2±4.0</td>
<td>1.50±0.12</td>
</tr>
<tr>
<td>2</td>
<td>AOM+O12+BC50</td>
<td>35.5±3.7</td>
<td>1.42±0.17</td>
</tr>
<tr>
<td>3</td>
<td>AOM+O12+BC200</td>
<td>35.4±3.3</td>
<td>1.41±0.18</td>
</tr>
<tr>
<td>4</td>
<td>AOM+P3O9</td>
<td>33.3±2.6</td>
<td>1.34±0.18</td>
</tr>
<tr>
<td>5</td>
<td>AOM+P3O9+BC50</td>
<td>33.2±4.0</td>
<td>1.30±0.16</td>
</tr>
<tr>
<td>6</td>
<td>AOM+P12</td>
<td>32.4±3.7</td>
<td>1.32±0.22</td>
</tr>
<tr>
<td>7</td>
<td>AOM+P12+BC50</td>
<td>31.6±3.5</td>
<td>1.31±0.15</td>
</tr>
</tbody>
</table>

Values represent mean ± SD.

*Significantly different from the AOM+O12 group; P < 0.01.
*Significantly different from the AOM+O12+BC50 group; P < 0.01.
*Significantly different from the AOM+O12+BC50 group; P < 0.01.
*Significantly different from the AOM+O12+BC50 group; P < 0.05.

**Fig. 3. ras mRNA expression in colonic mucosa of AOM-treated rats in each group.** Total RNA was isolated from the colonic mucosa, fractionated through 1% agarose-formaldehyde gels, transferred to nylon membranes and hybridized with $^{32}$P-labelled probes for ras (upper bands) and GAPDH (lower bands).
of 50 mg/kg body weight/day to 35.1 nmol/g at 200 mg/kg/day of β-carotene). However, no significant increases in retinol or retinyl ester concentrations were observed in colonic mucosa following β-carotene administration. This suggests that no significant amount of β-carotene was converted to retinoids in the mucosa. Thus, it is likely that β-carotene itself suppressed the development of ACF. The result also suggests that 4.6 nmol/g mucosa of β-carotene is enough concentration to suppress the development of ACF. β-Carotene has been shown to decrease adenylate cyclase activity (35), to enhance gap junction communication (36) via inducing connexin43 gene (37) and to inhibit DNA adduct formation (38). Very recently, Muto et al. in our laboratory have found that β-carotene reduces the expression of epidermal growth factor receptor in dysplastic uterine cervical cells (39), resulting in the growth arrest of the transformed cells. In addition, β-carotene inhibits tumour promoter-induced hyperphosphorylation of cytoskeletal proteins (40). Further studies will still be necessary to elucidate the mechanism of the antitumorigenic effect of β-carotene on the development of colon cancer.

These mechanisms by which β-carotene may act may be distinct from the proposed antitumorigenic mechanism of n-3 PUFAs. n-3 PUFAs may work by altering prostaglandin (PG) synthesis. PGs, particularly the type-2 series, are believed to be involved in colon carcinogenesis, since increased PG concentrations have been found in colon cancer tissue (5,41). PG_E2 induces hyperploration in colonic mucosa (42) and inhibitors of PG synthesis, such as indomethacin, suppress colon carcinogenesis in rats (43-45). n-3 PUFA has been reported to inhibit the production of the type-2 series of prostanoids, including PGE_2, from arachidonic acid (46-48). In fact, the PGE_2 concentrations in colonic mucosa was decreased by perilla oil (11). Another possible mechanism by which n-3 PUFA may act is an alteration in the fatty acid composition of colonic mucosal membranes. Oral administration of n-3 PUFA induced a selective incorporation of n-3 PUFAs and a competitive exclusion of n-6 PUFAs from the membrane phospholipid pools (11). The changes in the ratio of n-3 to n-6 PUFAs in the membrane could affect the functions of the membrane itself and/or of membrane bound receptors, such as epidermal growth factor receptor (49). Therefore, such change in membrane compositions could alter the sensitivity of cells to growth and carcinogenic stimuli (50).

The different mechanisms by which β-carotene and n-3 PUFA act may explain their synergistic effect on the suppression of development of colonic ACF. Further studies are currently underway to elucidate the mechanisms of the action of β-carotene and perilla oil. We are also preparing for a clinical trial of an interventional study to prevent colon cancer with the uses of β-carotene and/or perilla oil.

References


