Reduction in formation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced aberrant crypt foci in the rat colon by docosahexaenoic acid (DHA)

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Docosahexaenoic acid (DHA), a major component of fish oil, suppresses the formation and growth of aberrant crypt foci induced by 1,2-dimethylhydrazine and azoxymethane. In the present study we examined the effects of intragastric gavage administration of DHA on the yield of rat colonic aberrant crypt foci due to treatment with a heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), which induces colon cancer in male F344 rats and is considered to be a possible human colon carcinogen. Male F344 rats were given a standard diet (AIN-76A) and received 10 doses of PhIP (75 mg/kg body wt, by intragastric intubation, on days 1–5 and 8–12) with or without intragastric application of 1 ml DHA 4 h prior to each carcinogen treatment, followed by further DHA dosing. The numbers of PhIP-induced aberrant crypt foci per colon after 4 and 12 weeks DHA administration were significantly reduced to 47 and 38% respectively of the values obtained when PhIP alone was used. The mean number of aberrant crypts per focus was also decreased by DHA treatment. At week 4 the PhIP–DNA adduct levels in the colon of rats from the PhIP+DHA group were approximately two thirds of the PhIP group value. The results thus suggest that DHA exerts a preventive effect on PhIP-induced colon carcinogenesis.

Introduction

Epidemiological studies have shown that dietary factors play an important role in the etiology of human colon cancer. Diets rich in fat are generally associated with a high risk (1), while high intake of fish and seafood is associated with a low risk (2). Consistent with these epidemiological data, experiments with rodents have demonstrated that diets high in corn oil promote (3), whereas these containing large amounts of fish oils do not promote, but rather inhibit, azoxymethane (AOM)−induced colon carcinogenesis in rats (4,5). Fish oils are rich in polyunsaturated ω3 fatty acids, such as eicosapentaenoic acid (EPA, C20:5, ω3) and docosahexaenoic acid (DHA, C22:6, ω3), and dietary EPA itself has been reported to suppress AOM-induced rat colon carcinogenesis (6). DHA has also been shown to decrease the formation and growth of aberrant crypt foci (ACF), which are putative preneoplastic lesions, induced by 1,2-dimethylhydrazine (DMH) and AOM in the rat colon (7,8). Recently it was further demonstrated that DHA can suppress the number of colon carcinomas induced by AOM in rats (9). Moreover, perilla oil, rich in ω3-linolenic acid, an ω3 fatty acid precursor of EPA, was found to decrease N-methyl-N-nitrosourea (MNU)-induced rat colon carcinogenesis (10). Thus it is very clear that ω3 polyunsaturated fatty acids can suppress rat colon carcinogenesis induced by chemical carcinogens such as AOM, DMH and MNU. However, these carcinogens are all methylating agents and it is still unknown whether ω3 fatty acids can similarly inhibit colon carcinogenesis induced by other types of carcinogens, especially naturally occurring forms.

It is well known that human beings are chronically exposed to food-derived mutagenic and carcinogenic heterocyclic amines in their daily life (11). 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), one such heterocyclic amine which is particularly abundant in cooked meat and fish (11,12), undergoes metabolic activation by CYP1A2 to produce a N-hydroxyamine derivative. This proximate compound is further converted to the ultimate N-aceotxy or N-sulfate forms by N-acetyltransferase and sulfotransferase respectively (13–15), which then covalently bind to DNA, yielding N-(deoxyguanosin-8-yl)-PhIP as the major adduct (16,17). Gas chromatography/mass spectrometry and 32P-postlabeling have demonstrated the presence of PhIP−DNA adducts in DNA samples from human colon (18). PhIP primarily induces colon tumors in male F344 rats (19). The available data thus suggest that it is likely to play a role in human colon cancer, so that examination of whether the ω3 polyunsaturated fatty acid DHA might suppress colon carcinogenesis induced by PhIP is very pertinent.

PhIP has been reported to induce ACF in F344 rats (20,21). Thus the present relatively short-term study of the effects of DHA on PhIP carcinogenicity was conducted. Here we document that DHA reduces the number and size of ACF caused by PhIP. In addition, a suppressive effect of DHA on formation of PhIP−DNA adducts in the colon of rats is described.

Materials and methods

Chemicals

DHA was purified as the DHA ethyl ester at Sagami Chemical Research Center (Sagamihara, Japan) to >99% purity with <1% EPA as a contaminant. No antioxidants were added to the DHA preparation, which was stored under anaerobic conditions at −20°C in the dark. PhIP-HCl was obtained from the Nard Institute (Osaka, Japan).

Animals

Male F344 rats (Charles River Japan Inc., Atsugi, Japan) were used at 6 weeks of age. The animals were housed in plastic cages in an air conditioned room with a 12 h light–dark cycle and were provided with standard diet (AIN-76A; Dyets Inc., Bethlehem, PA) and water ad libitum. The animals were weighed weekly throughout the experiment.

Abbreviations: AOM, azoxymethane; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ACF, aberrant crypt foci; DMH, dimethylhydrazine; MNU, N-methyl-N-nitrosourea; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; DHLA, dihomo-γ-linolenic acid; AA, arachidonic acid; ACs, aberrant crypts.
PhIP-HCl was dissolved in water at a concentration of 15 mg/ml. This solution was given to male F344 rats at a dose of 5 ml/rat body wt, days 1–5 and 8–12. The animals were also given either 1 ml/rat was given to male F344 rats at a dose of 5 ml/kg body wt by daily i.g. gavage. PhIP-DNA adducts were obtained by 32P-postlabeling methods using modified adduct intensification conditions, in which 32P-labeled DNA samples were further treated with nuclease P1 and phosphodiesterase I to form mononucleotides, as previously described by Fukutome et al. (25).

Quantification of PhIP-DNA adducts in the colon mucosa
PhIP-DNA adducts in the colon mucosa were quantified by 32P-postlabeling methods under modified adduct intensification conditions, in which 32P-labeled DNA samples were further treated with nuclease P1 and phosphodiesterase I to form mononucleotides, as previously described by Fukutome et al. (25).

Statistical analysis
The significance of intergroup differences in values was determined using the t-test and a P value of <0.05 was regarded as significant.

Results
In the first 2 weeks the body weight gains of rats almost ceased with PhIP treatment and subsequently, although the body weights started to increase again, they remained depressed, with a 16–17% reduction evident at week 4 and a 13–14% reduction at week 12. Administration of DHA had no effect on body weight gain and did not cause diarrhea.

ACF were found in the colons of all the animals treated with PhIP and were located mainly in the middle (~60%) and distal (~40%) segments. Only 1–2% were observed in the proximal colon and ACF was extremely rare in the rectum. As shown in Table I, the numbers of PhIP-induced ACF and total numbers of aberrant crypts (ACs) per colon were significantly smaller in the group given PhIP plus DHA than in the PhIP group. The numbers of PhIP-induced ACF per colon after 4 weeks and 12 weeks of DHA treatment were reduced to 47 and 38% of the respective PhIP group values. The average numbers of ACs per focus were also smaller in the PhIP-DHA group. No development of ACF was observed in rats treated with DHA alone.

Table I shows the incidences of PhIP-induced ACF of different sizes, classified according to the number of ACs in each focus. In the PhIP-DHA group, the percentages of rats having ACF with two to four crypts at week 4 and three to 16 crypts at week 12 were less than those in the PhIP group. At week 4 ACF comprising four ACs were observed in two rats of the PhIP group, but in none of the PhIP-DHA group animals. At week 12 the incidences of ACF consisting of four and five crypts were significantly decreased by DHA treatment (P < 0.05). A total of six ACF consisting of ≥6 ACs (maximum 16 ACs per colon) were observed in four of nine rats in the PhIP group, but only one such large ACF (eight ACs) was found in eight rats in the PhIP-DHA group. Figure 1 shows histograms of the sizes of the ACF. The number of ACF in each size class was less in the PhIP-DHA group than in the PhIP group at both week 4 and week 12. The number of large ACF consisting of ≥3 in the PhIP-DHA group at week 4 were 0.4 ± 0.5, as compared with 1.3 ± 1.0 (P < 0.05) in the PhIP group. The number of large ACF consisting of ≥5 ACs at week 12 were also lower in the PhIP-DHA group than the PhIP group (0.5 ± 0.5 versus 2.2 ± 1.1, P < 0.01).

Data for the plasma levels of four fatty acids are shown in Table III. DHA treatment significantly increased the DHA and EPA levels 7- to 4-fold and 80- to 30-fold respectively and significantly decreased the AA level to 1/4–1/3 of that in the PhIP group. The DHLA level was not significantly affected by the DHA treatment.

Figure 2 shows the DNA adduct pattern in the colon of a rat given PhIP, a single spot corresponding to N-(deoxyguanosin-8-y1)-PhIP S′-monophosphate (5′-pDG-C8-PhIP) being revealed by the 32P-postlabeling method under modified adduct intensification conditions. Quantitation of PhIP-DNA adducts in colon mucosa at week 4 demonstrated a decrease from 14.9 ± 7.5/10⁶ nucleotides in the PhIP alone case to 9.6 ± 2.9/10⁶ nucleotides in the PhIP-DHA group.

Discussion
PhIP has been reported to induce ACF in the colon of rats: 1–6 ACF/rat were found after feeding of 0.04–0.05% PhIP in the diet for 4–12 weeks (20,21), and oral gavage of 50 mg PhIP/kg body wt on alternate days for 2 weeks resulted in development of 3.3 ± 1.8 ACF/rat at week 14 (26). Compared with previous studies, the experimental conditions (75 mg/kg body wt, days 1–5 and 8–12, a total of 10 doses) adopted in the present study were shown to induce ACF quite efficiently, the yields being 15.1 ± 3.3 at week 4 and 21.6 ± 5.7 at week 12, allowing demonstration of a significant decrease by DHA administration. The fact that both the incidence and number of large ACF were reduced in the DHA-treated group suggests that DHA inhibited not only the induction of ACF, but also their growth in the colon of rats treated with PhIP.

As previously reported (7–9), the level of AA was reduced in the blood plasma of rats treated with DHA, while DHA

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Reduction in PhIP-induced aberrant crypt foci by DHA

Table II. Effect of DHA on the incidence of PhIP-induced ACF of different sizes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experimental period (weeks)</th>
<th>Incidencea of ACF with varying numbers of crypts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of crypts per focus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>PhIP + Water</td>
<td>4</td>
<td>9/9</td>
</tr>
<tr>
<td>PhIP + DHA</td>
<td>4</td>
<td>9/9</td>
</tr>
<tr>
<td>PhIP + Water</td>
<td>12</td>
<td>9/9</td>
</tr>
<tr>
<td>PhIP + DHA</td>
<td>12</td>
<td>8/8</td>
</tr>
</tbody>
</table>

a No. of rats with ACF of each size/total no. of rats.

b Significantly different from the corresponding control value at \( P < 0.05 \).

Table III. Effects of DHA on levels of four fatty acids in blood plasma of PhIP-treated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experimental period (weeks)</th>
<th>No. of samples</th>
<th>Fatty acids (µg/ml)a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DHLA (C20:3, ( \omega_6 ))</td>
</tr>
<tr>
<td>PhIP + Water</td>
<td>4</td>
<td>4</td>
<td>9.6 ± 2.8</td>
</tr>
<tr>
<td>PhIP + DHA</td>
<td>4</td>
<td>4</td>
<td>11.0 ± 2.6</td>
</tr>
<tr>
<td>PhIP + Water</td>
<td>12</td>
<td>4</td>
<td>14.2 ± 16.7</td>
</tr>
<tr>
<td>PhIP + DHA</td>
<td>12</td>
<td>4</td>
<td>16.7 ± 5.8</td>
</tr>
</tbody>
</table>

a The data presented are averages ± SD values.
b Significantly different from the corresponding control value at \( P < 0.001 \) and \( P < 0.01 \) respectively.

d and EPA were increased. The expression of cyclooxygenase 2 and prostaglandin \( \mathrm{E}_2 \) has been shown to be clearly elevated in colon tumors of rats and humans (6,27–29) and DHA is known to be an inhibitor of prostaglandin synthesis (30,31). Therefore, the reduction in the AA level and elevation of DHA and EPA due to DHA treatment might be expected to result in decreased prostaglandin \( \mathrm{E}_2 \) levels in the colon mucosa and suppression of colon carcinogenesis. This would presumably depend on altered growth to some extent.

Polyunsaturated fatty acids, including EPA and DHA, have also been found to inhibit the mutagenic actions of heterocyclic amines, 2-amino-3-methylimidazo[4,5-f]quinoline, 2-amino-3,8-dimethylimidazo[4,5-f]quinoline and PhIP in \textit{in vitro} bacterial mutation assays, using \textit{Salmonella typhimurium} TA98 with S9 mix (32). This suggests that the reduction in the level of PhIP–DNA adducts in the colon of rats given DHA, shown in the present study, is due to inhibition of metabolic activation of PhIP by DHA, although enhancement of removal of PhIP–DNA adducts from colon mucosa also cannot be ruled out at present. Concerning the reduction in PhIP–DNA adducts, there is another possibility, that DHA decreased PhIP absorption in the gastrointestinal tract under the conditions used in the present study. However, the contents of unmetabolized PhIP and 4'-hydroxy-PhIP in the 24 h urine of rats given PhIP 4 h after administration of DHA or water were not significantly different between the two groups: excretion of unmetabolized PhIP was 1.22 ± 0.22% in the DHA group and 1.29 ± 0.12% in the water group, with values of 1.96 ± 0.55% and 1.53 ± 0.30% respectively for the metabolite 4'-hydroxy-PhIP (unpublished data). Therefore, it seems that DHA administration in this study did not affect the absorption
of PhIP and the reduction in adduct formation was therefore due to another mechanism.

In experimental colon carcinogenesis induced by DMH and AOM, mutations in the Ki-ras gene have been frequently observed in tumors and ACF (33–37). However, in the case of PhIP-induced colon carcinogenesis, involvement of Ki-ras gene mutations is reported to be rare (38). Instead, specific growth of ACF induced by DMH (7), the beneficial influence demonstrated that DHA also suppresses the formation and growth of amount and source of dietary fat.

Acknowledgements

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