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Reduction in Formation and Growth of 1,2-Dimethylhydrazine-induced Aberrant Crypt Foci in Rat Colon by Docosahexanoic Acid[1]

Mami Takahashi,[2] Toshinari Minamoto, Naoyuki Yamashita, Kazunaga Yazawa, Takashi Sugimura, and Hiroyasu Esumi

Biochemistry Division, National Cancer Center Research Institute, Tokyo [M. T., T. M., N. Y., T. S., H. E.], and Sagami Chemical Research Center, Kanagawa [K. Y.], Japan

ABSTRACT

The effect of intragastric gavage administration of docosahexaenoic acid (DHA) on the formation of 1,2-dimethylhydrazine (DMH)-induced aberrant crypt foci in rat colon was investigated. Male F344 rats were treated three times s.c. with 20 mg/kg of DMH and were given either 0.7 ml of DHA or water intragastrically 5 times a week for 4, 8, or 12 weeks from the day before the first carcinogen treatment. The numbers of DMH-induced aberrant crypt foci per colon after 4, 8, and 12 weeks of DHA treatment were approximately 40% of those in the respective control groups, and the differences were statistically significant. The numbers of foci reached plateau levels at 8 weeks in both the DHA-treated and control groups. The mean number of aberrant crypts per focus was also significantly smaller in the group given DHA than that in the control group at each time. These results suggest that DHA suppresses the formation and growth of aberrant crypt foci and has a preventive effect on 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, particularly animal fat, or poor in certain fibers are generally associated with a high risk for colon cancer (1). However, epidemiological studies and several studies in animal models have shown that fish oils do not promote (2) but, instead, inhibit (3–6) colon carcinogenesis. For the prevention of colon cancer, it is very important to know which component of fish oil is effective in preventing colon carcinogenesis and the mechanism of its preventive effect. Fish oils are rich in highly polyunsaturated ω3 fatty acids such as EPA[3] (C20:5, ω3) and DHA (C22:6, ω3). Dietary EPA is reported to suppress azoxymethane-induced rat colon carcinogenesis (5), and DHA-enriched fish oil has been suggested to be more effective than EPA-enriched fish oil in preventing colon carcinogenesis (6).

Recently, aberrant crypts, which are putative preneoplastic lesions, were observed at high frequency in the colonic mucosa of patients with colon cancer (7, 8) and of rats and mice treated with colon carcinogens (9–12). The effects of several food components and of an inhibitor of colon carcinogenesis on aberrant crypt focus formation have been analyzed in animal models (13–16). Aberrant crypt foci and their growth are thought to be useful indices of the effects of carcinogens and agents preventing carcinogenesis in the colon. In the present paper, we report that highly purified DHA suppressed the formation and growth of aberrant crypt foci induced by DMH in rats.

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[2] To whom requests for reprints should be addressed, at Biochemistry Division, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan.

[3] The abbreviations used are: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DMH, 1,2-dimethylhydrazine; AA, arachidonic acid.

MATERIALS AND METHODS

Chemicals. DHA was purified as DHA-ethyl ester at Sagami Chemical Research Center (Kanagawa, Japan). The DHA-ethyl ester preparation was more than 97% pure and contained less than 3% EPA as a contaminant. No antioxidant was added to the DHA preparation, which was stored in sealed ampules under anaerobic conditions at −20°C in the dark. DMH dihydrochloride was from Tokyo Chemical Industry (Tokyo, Japan).

Animals. Male F344 rats (Charles River Japan, Inc., Kanagawa, Japan) that were about 6 weeks old were used. The animals were housed in wire cages in an air-conditioned room with a 12-h light-dark cycle and were given CE-2 pellet diet (CLEA Japan, Tokyo) and water *ad libitum*. The animals were weighed weekly throughout the experiment.

Carcinogen and DHA Treatments. In experiment 1, 57 animals were divided into two groups and treated s.c. with DMH dihydrochloride at a dose of 20 mg/kg body weight 3 times at 3-day intervals. The DMH dihydrochloride was administered as a solution in 1 mM EDTA, adjusted to pH 6.5 with NaOH. The two groups were given 0.7 ml of DHA and 0.7 ml of water, respectively, 5 times a week by gastric intubation starting the day before carcinogen treatment. One-third of the animals in each group were sacrificed 4, 8, and 12 weeks after the first treatment with DMH.

In experiment 2, 40 rats were divided into four groups. DMH dihydrochloride was injected in the same way as in experiment 1. From the day before the first carcinogen treatment, groups A and B were treated intragastrically with 0.7 ml of water, and groups C and D with 0.7 ml of DHA 5 times a week. Two days after the last carcinogen treatment, groups B and C were switched to treatments with DHA and water, respectively. The former period (consisting of the day before, the period during, and the day after carcinogen treatment) was termed the initiation stage, and the latter period (starting 2 days after the last carcinogen treatment) was termed the postinitiation stage. All animals were sacrificed 4 weeks after the first treatment with DMH.

Analysis of Aberrant Crypts. The colon was removed, slit open from the anus to the cecum along the longitudinal axis, spread flat between sheets of filter paper, and fixed in buffered 10% formalin. Then it was stained with 0.2% methylene blue in saline by the procedure of Bird (9) to observe aberrant crypts. The number of aberrant crypt foci per colon, the number of aberrant crypts in each focus, and the location of each focus were determined by microscopy at a magnification of ×40. To determine the distribution of aberrant crypt foci, we defined the rectum as the segment 2 cm proximal to the anus and divided the remaining colon into three segments of about 6 cm length, the distal colon, the middle colon, and the proximal colon.

Analysis of Serum Cholesterol and Plasma Fatty Acid Concentrations. Animals were starved for 12 h before sacrifice, and blood was collected by heart puncture. The serum cholesterol and plasma levels of four fatty acids, dihomo-γ-linolenic acid, AA, EPA, and DHA, were determined enzymatically (17) and by gas chromatography, respectively.

Statistical Analysis. The significance of the differences between values for different groups was analyzed by Student's *t* test, and a *P* value of <0.05 was regarded as significant.

RESULTS

The changes in mean body weights of the two groups in experiment 1 are shown in Fig. 1. In the early stage of the experiment, the administration of DHA caused a reduction in body weight, probably because the dose of DHA was slightly excessive for young rats, with an average body weight of about 113 g. Later, the body weights of DHA-treated rats increased in parallel with those of control animals.

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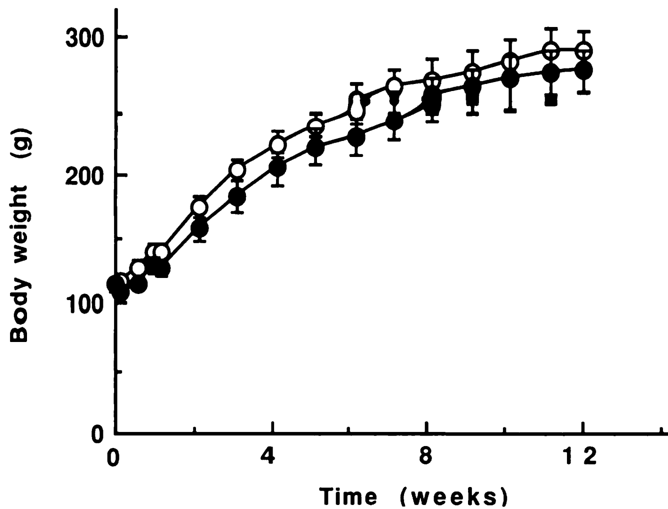

Fig. 1. Mean body weights of DMH-treated rats given DHA or water intragastrically in experiment 1. *Bars*, SE; ●, DHA; ○, water.

The numbers of aberrant crypt foci, total numbers of aberrant crypts per colon, and mean numbers of aberrant crypts per focus in the two groups in experiment 1 are shown in Table 1. Aberrant crypt foci were found in the colons of all animals treated with DMH. Aberrant crypt foci were located mainly in the distal colon with some in the middle colon, a few in the rectum, and very few in the proximal colon. The numbers of DMH-induced aberrant crypt foci per colon after 4, 8, and 12 weeks of DHA treatment were approximately 40% of those in the respective control groups, and the differences in their values between the two groups were significant. The numbers of foci reached plateau levels at 8 weeks in both DHA-treated and control groups, so the plateau level itself was reduced by DHA treatment. The mean size of aberrant crypt foci, measured as the number of crypts per focus, was also significantly smaller in the group given DHA than in the control group at each time point.

Fig. 2 shows histograms of the sizes of aberrant crypt foci, measured as the number of aberrant crypts in each focus, in experiment 1. The numbers of foci of each size were significantly less in the DHA-treated group than in the control group. In the control group, the number of foci composed of a single aberrant crypt decreased markedly with time, and the peak of the histogram shifted to the right, namely to larger sizes of foci. But in the DHA-treated rats, the peak of the histogram remained at one aberrant crypt per focus, even in week 12.

In experiment 2, the effect of DHA treatment during the initiation and/or postinitiation stage on DMH-induced aberrant crypt foci was investigated. Results are summarized in Table 2. The numbers of foci per colon were significantly lower in both group C (treated with DHA during the initiation period) and group B (treated with DHA during the postinitiation period) than in group A (without DHA treatment). The number of foci in group D (treated with DHA during both the initiation and postinitiation periods) was lower than those in groups B and C. The mean size of foci was reduced in group B, as in group D. Administration of DHA during the initiation period did not reduce significantly the mean size of foci.

Data on the plasma levels of four fatty acids and the serum level of cholesterol in experiment 1 are shown in Tables 3 and 4, respectively. DHA treatment increased the DHA and EPA levels 1.5–1.8-fold but decreased the AA level to about a one-half that in the control group and did not affect the dihomo-γ-linolenic acid level significantly. The serum cholesterol level in the DHA-treated group was three-quarters of that in the control group.

DISCUSSION

In the present study, we showed that DHA treatment reduced the formation and growth of DMH-induced aberrant crypt foci in rat colon. Narisawa *et al.* (6) suggested that DHA-enriched fish oil (DHA 74%, EPA 12.7%) inhibits colon carcinogenesis induced by methylnitrosourea in rats, and Minoura *et al.* (5) showed that diet containing 4.7% EPA (91% pure) suppresses carcinogenesis in rat colon. In the present study, highly purified DHA (>97%) inhibited colon carcinogenesis by suppressing both the formation and growth of the putative preneoplastic lesions.

In experiment 1, the administration of DHA overlapped carcinogen treatment and reduced the plateau level of the number of foci, indicating that DHA could affect the metabolism of the carcinogen, resulting in a reduction of induction of aberrant crypt foci in the initiation stage. This finding is consistent with reports that the levels of drug-metabolizing enzymes are influenced by dietary lipids (18, 19). In addition, the low proportion of foci of large sizes in DHA-treated groups may indicate that DHA affected the proliferation and/or cell-cell interactions of epithelial cells of the colon mucosa directly or indirectly, suppressing the induction and growth of aberrant crypt foci at the postinitiation stage. Indeed, we found in experiment 2 that DHA administration overlapping carcinogen treatment decreased the induction of foci and that DHA administration during the postinitiation period reduced the growth of foci as well. These findings are consist

Table 1 *Effect of DNA on DMH-induced aberrant crypt foci in rat colon (experiment 1)*

Treatment (i.g.)[a]	Experimental period (weeks)	Incidence[b]	No. of foci/colon[c] (average ± SE)	No. of aberrant crypts (average ± SE)	Mean no. of aberrant crypts/focus (average ± SE)
Water	4	11/11	122.1 ± 35.3 (100%)	229.5 ± 69.2 (100%)	1.88 ± 0.22
DHA	4	10/10	42.4 ± 18.7[d] (34.7%)	69.8 ± 35.7[d] (30.4%)	1.60 ± 0.20[e]
Water	8	10/10	147.2 ± 54.2 (100%)	373.5 ± 143.9 (100%)	2.53 ± 0.17
DHA	8	8/8	59.1 ± 18.8[e] (40.1%)	134.4 ± 50.9[d] (36.0%)	2.22 ± 0.30[f]
Water	12	10/10	152.5 ± 48.0 (100%)	455.8 ± 150.3 (100%)	2.98 ± 0.23
DHA	12	8.8	60.9 ± 21.9[d] (39.9%)	162.9 ± 53.5[d] (36.5%)	2.70 ± 0.26[g]

[a] Intragastric administration.
[b] No. of rats with aberrant crypt foci/total rats.
[c] The total number of foci in all four regions examined.
[d–g] Significantly different from the corresponding control values at $P < 0.001$, $P < 0.01$, $P < 0.02$, and $P < 0.05$, respectively.

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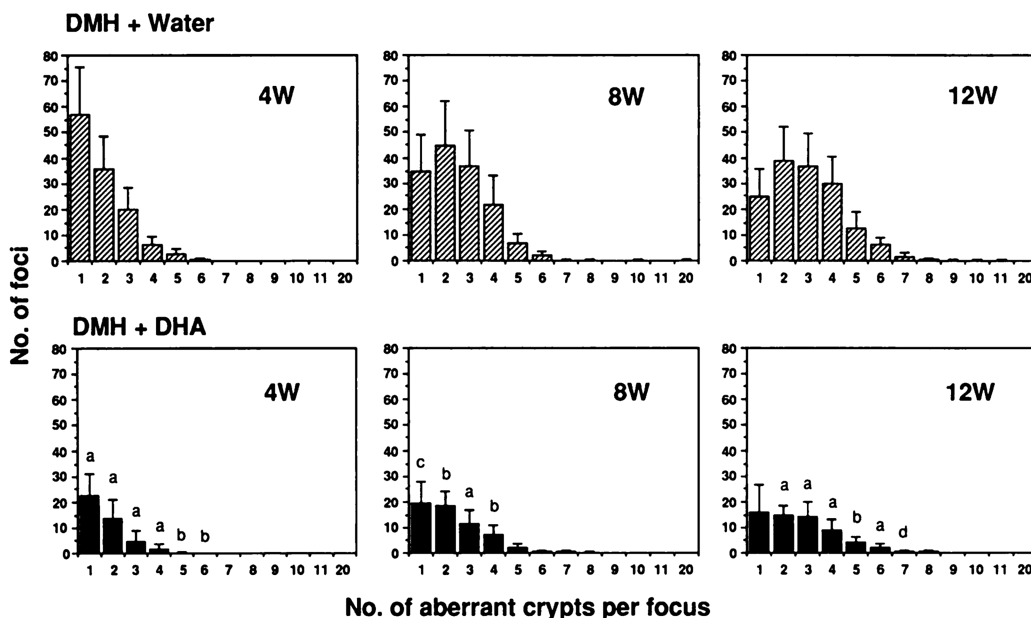

Fig. 2. Size distributions of DMH-induced aberrant crypt foci in the colon of rats treated intragastically with DHA or water in experiment 1. *Columns*, averages; *bars*, SE; *a, b, c, d*, significantly different from the corresponding control value at $P < 0.001$, $P < 0.01$, $P < 0.02$, and $P < 0.05$, respectively.

Table 2 *Effects of DHA treatment during initiation and postinitiation stages*[a] *on DMH-induced aberrant crypt foci in rat colon (experiment 2)*

Group	Treatment (i.g.)[b]	Incidence[c]	No. of foci/colon[d] (average ± SE)	No. of aberrant crypts (average ± SE)	Mean no. of aberrant crypts/focus (average ± SE)
A	Water	10/10	151.7 ± 64.2[g]	283.0 ± 125.5[g]	1.86 ± 0.13[g]
B	Water + DHA[e]	10/10	99.9 ± 25.0[h]	156.2 ± 35.1[h]	1.57 ± 0.10[h]
C	DHA + water[f]	10/10	90.1 ± 19.0[h]	159.0 ± 40.6[h]	1.76 ± 0.11[g]
D	DHA	10/10	66.9 ± 19.7[i]	104.4 ± 31.0[i]	1.56 ± 0.10[h]

[a] In the present study, initiation and postinitiation stages mean the periods of carcinogen and postcarcinogen treatment.
[b] Intragastric administration.
[c] No. of rats with aberrant crypt foci/total rats.
[d] The total number of foci in all four regions examined.
[e] Animals were given DHA intragastrically from 2 days after carcinogen treatment until the end of the experiment.
[f] Animals were given DHA intragastrically the day before, during, and the day after carcinogen treatment.
[g–i] Averages in the same vertical column that do not share a common superscript are significantly different at $P < 0.05$.

Table 3 *Effects of DHA on four fatty acids in blood plasma of DMH-treated rats (experiment 1)*

Treatment (i.g.)[a]	Experimental period (weeks)	No. of samples	Fatty acid (μg/ml) (average ± SE) DHLA (C20:3,ω6)	AA (C20:4,ω6)	EPA (C20:5,ω3)	DHA (C22:6,ω3)
Water	4	7	3.6 ± 0.7	231.9 ± 31.0	18.6 ± 3.9	56.8 ± 13.2
DHA	4	8	3.8 ± 0.5	122.5 ± 22.7[b]	31.1 ± 8.6[c]	103.5 ± 35.0[d]
Water	8	9	5.4 ± 1.2	253.8 ± 26.6	30.5 ± 4.2	60.6 ± 7.0
DHA	8	4	5.3 ± 1.4	109.6 ± 10.0[b]	46.2 ± 23.5	101.7 ± 67.1
Water	12	9	6.1 ± 1.2	253.4 ± 41.3	24.3 ± 6.8	60.6 ± 12.5
DHA	12	8	5.4 ± 0.9	99.4 ± 5.8[b]	41.4 ± 12.6[c]	105.0 ± 54.6

[a] Intragastric administration.
[b–d] Significantly different from the corresponding control value at $P < 0.001$, $P < 0.01$, and $P < 0.02$, respectively.

with a report that a diet high in fish oil reduces colon tumors in rats in either the initiation and/or postinitiation stage of carcinogenesis (4). Because some aberrant crypt foci exhibit dysplasia (20), which is an important feature of precursor lesions of colon cancer (21), there is another possibility that DHA decreased the degree of dysplasia. A detailed morphological study of these lesions will help clarify this point.

We observed reductions in serum cholesterol and AA levels and increases in DHA and EPA levels in DHA-treated rats. This finding is consistent with reports that fish oil reduces the cholesterol and AA contents of rat serum and liver lipid fractions (22–24). In general, dietary fats that contain linoleic acid, a precursor of prostaglandins,

Table 4 *Effect of DHA on total cholesterol in serum of rats treated with DMH (experiment 1)*

Treatment (i.g)[a]	Experimental period (weeks)	No. of samples	Total cholesterol (mg/dl) (average ± SE)
Water	4	6	38.7 ± 2.9
DHA	4	7	29.9 ± 4.6[b]
Water	8	5	41.2 ± 3.9
DHA	8	6	30.7 ± 3.4[b]
Water	12	6	38.3 ± 4.8
DHA	12	8	30.4 ± 5.1[c]

[a] Intragastric administration.
[b,c] Significantly different from the corresponding control value at $P < 0.01$ and $P < 0.02$, respectively.

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are effective in promoting tumorigenesis in animals (25). On the other hand, indomethacin, which inhibits prostaglandin synthesis, inhibits tumor promotion in rat colon (26, 27). DHA is also known to be a strong inhibitor of prostaglandin synthesis (28). Therefore, the mechanisms underlying the inhibitory effect of DHA on the formation of aberrant crypt foci may be linked in part to the inhibition of prostaglandin synthesis from AA and reduction of the AA content itself.

There is a recent report that EPA and DHA have protective effects on the transformation of cultured cells and that the addition of EPA or DHA to the culture medium results in extensive remodeling of the molecular species of phospholipids of the cells (29). The mechanism of the effect of DHA could also involve changes in cell surface receptors by alteration of the fluidity of cellular membranes, alteration of immune responses, effects on cholesterol and bile acid levels, and radical scavenging.

Dietary factors are closely associated with human cancer risk. Thus further studies on both carcinogenic factors and anticarcinogenic factors in foods are important for the prevention of colon cancer.

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