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Effects of Dietary n–3-to-n–6 Polyunsaturated Fatty Acid Ratio on Mammary Carcinogenesis in Rats

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Abstract: We investigated the effects of the dietary n–3-to-n–6 polyunsaturated fatty acid (PUFA) ratio (n–3/n–6 ratio) on mammary carcinogenesis induced by 7,12-dimethylbenz[a]anthracene in rats by feeding them several types of dietary fat with a fixed PUFA-to-saturated fatty acid ratio. Dietary fat was fed to the rats as 10% of the total feed weight, starting two weeks before the initiation. An increase in the n–3/n–6 ratio did not suppress the incidence or reduce the latency of mammary tumor development. The number and weight of mammary tumors per tumor-bearing rat tended to be large in the group with an n–3/n–6 ratio of 7.84 compared with those in the other groups. As the n–3/n–6 ratios were elevated, the total number and weight of tumors increased gradually. The prostaglandin E_2 (PGE_2) concentration in mammary tumor tissue was markedly low in the group with an n–3/n–6 ratio of 1.03 compared with the group with an n–3/n–6 ratio of 0.01. In addition, PGE_2 concentrations were almost constant when n–3/n–6 ratios were >1.03. These results suggested that the increase in the n–3/n–6 ratio of dietary fat with the fixed PUFA-to-saturated fatty acid ratio cannot suppress the mammary carcinogenesis but can promote development of tumors, despite reduced PGE_2 concentration in the tumor.

Introduction

The quantity and quality of fat ingested are representative dietary factors that greatly affect the development of mammary and colonic tumors (1–4). Epidemiologic studies involving Greenlanders, who consume large amounts of fat, revealed relationships between not only the amount of fats but also the type of fatty acids ingested and the development of tumors (1,2). The results of experiments using animals also suggested that development of mammary tumors, which commonly are observed in Europeans, was promoted by an increase in the amount of fat ingested (3,4). Thus the relationship between the development of tumors and quality of fat ingested has become a subject of research (5–21).

Mammary carcinogenesis is promoted by 18:2, one of the n–6 polyunsaturated fatty acids (PUFAs) (5,6). It was reported that the incidence of mammary tumors increased as a result of ingesting fat containing a high concentration of 18:2, but not as a result of ingesting fat containing the same concentration of medium-chain fatty acids or 18:1 (7). However, Ip and co-workers (8) indicated in their study, in which the 18:2 concentration of dietary fat was increased in a stepwise manner, that the promotional effect of dietary fat on 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary carcinogenesis was closely related to the level of 18:2. That is, the incidence and the number of mammary tumors increased linearly with increasing 18:2 concentration up to 4.4%; however, the effect of 18:2 on mammary carcinogenesis did not continue to change when the concentration increased beyond this point. In contrast, it has been verified by a number of studies (10–21) that when fish oil (FO)- or perilla oil-based diets, which contain large amounts of n–3 PUFA, were ingested, the incidence and the proliferation of mammary tumors were suppressed compared with cases in which plant oil containing large amounts of n–6 PUFA [i.e., safflower oil (SO) or corn oil (CO)] was ingested.

However, the various kinds of fat used in the above animal experiments differ not only in the kinds of PUFA they contain but also in the amounts of total PUFA, monounsaturated fatty acid (MUFA), and saturated fatty acid (SFA). Therefore, we cannot conclude that the suppression of the incidence of mammary tumors induced by the ingestion of fish oil is due only to the effect of n–3 PUFA. The reason for this is that FO contains larger amounts of SFA and MUFA than does SO, so the incidence of mammary tumors could be suppressed by the intake of SFA or MUFA (7,10). In the current study, we investigated the effects of dietary fat with different n–3/n–6 ratios on the development of mammary carcinogenesis induced by the administration of DMBA through administration of several types of dietary fat with constant PUFA-to-SFA (P/S) ratio, for which the n–3/n–6 ratio was increased in a stepwise manner, using FO and SO as sources of n–3 and n–6 PUFA, respectively.

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Methods

Animals and Diets

Twenty-eight-day-old female Sprague-Dawley rats (Tokyo Laboratory Animal Science, Tokyo, Japan) were used in all experiments. They were housed in an air-conditioned room (22 ± 1°C and 50 ± 10% humidity) under a 12:12-hour light-dark cycle. For adaptation to the experimental regimen, the rats were fed MF-2 rat chow (Oriental Yeast, Tokyo, Japan) and divided into experimental dietary groups to give the same values obtained in body weight in each of the groups. They were housed in individual cages. Semipurified diets were prepared daily by adding the oils to the other dietary components. The food was placed in the cages at 1700 and removed at 0900 the following morning to avoid the ingestion of lipid peroxides. The stock powder diet and test oils were stored separately at −30°C in dark bottles under nitrogen until use. The degree of lipid peroxidation of test oils was monitored by thiobarbituric acid-reactive substance (TBARS) assay (22), and no lipid peroxidation was detected. The rats were allowed water ad libitum. The semipurified diet contained 20% casein, 0.3% DL-methionine, 60% sucrose, 3.5% mineral mix (AIN-76), 1% vitamin mix (AIN-76), 0.2% choline bitartrate, 5% cellulose, and 10% (wt/wt) fat. The fats were prepared by mixing FO (sardine oil, Nihon Oil and Fats, Tokyo, Japan), SO (Nihon Oil and Fats), and coconut oil (CCO; Hayashi Chemicals, Tokyo, Japan). The fatty acid compositions of the dietary fats used in each experiment were analyzed by gas chromatography (Tables 1 and 2). Briefly, the dietary fats were transmethylated with boron trifluoride-methanol complex (23) and then analyzed on a gas chromatograph (model GC-12A, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a 0.25 mm × 40 m stainless steel capillary column (Silar 5CP, Chromatotec, Tokyo, Japan). Analysis was conducted at a column temperature of 200°C and inlet temperature of 250°C, with nitrogen gas (2.2 ml/min) as the carrier. A single dose of 15 mg of DMBA (Sigma Chemical, St. Louis, MO) dissolved in 1 ml of SO was given to each rat on Day 49 after birth. The rats were palpated daily to monitor tumor development.

Table 1. Fatty Acid Composition of Coconut, Safflower, and Fish Oil[a,b]

	Experiment 1			Experiment 2		
Fatty Acids	CCO	SO	FO	CCO	SO	FO
8:0	4.2	ND	ND	3.8	ND	ND
10:0	5.4	ND	0.1	5.4	ND	0.1
12:0	47.7	ND	0.2	49.1	ND	1.3
14:0	18.6	0.1	6.2	19.2	0.2	8.1
16:0	10.6	6.6	14.9	10.1	7.1	17.4
18:0	3.2	2.4	2.7	2.7	2.4	3.4
18:1n–9	8.5	14.7	14.7	8.2	13.8	14.6
18:2n–6	1.7	75.1	1.6	1.7	74.7	1.9
20:5n–3	ND	ND	16.4	ND	ND	16.3
22:6n–3	ND	ND	11.8	ND	ND	9.7
SFA	89.8	9.2	26.8	90.1	9.7	32.4
MUFA	8.5	15.0	30.9	8.2	14.0	27.1
PUFA	1.7	75.8	42.4	1.7	76.4	40.5
PUFA/SFA	0.02	8.25	1.58	0.02	7.91	1.25
n–3 PUFA	ND	0.4	36.7	ND	0.9	33.2
n–6 PUFA	1.7	75.1	3.7	1.7	74.7	4.0
n–3/n–6	0.00	0.01	9.87	0.00	0.01	8.40

a: Values are percentages, except for ratios.

b: Abbreviations are as follows: CCO, coconut oil; SO, safflower oil; FO, fish oil; ND, not detected; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 2. Fatty Acid Composition of Dietary Fats[a,b]

	Experiment 1				Experiment 2	
Fatty Acids	Group I	Group II	Group III	Group IV	Group A	Group B
8:0	2.8	0.6	0.7	0.5	2.1	1.3
10:0	2.7	1.2	0.8	0.7	2.4	1.3
12:0	20.8	12.1	8.7	7.6	21.7	11.8
14:0	8.1	8.0	8.2	8.1	8.4	8.9
16:0	8.1	11.7	13.6	13.9	8.4	13.4
18:0	2.6	2.6	2.5	2.5	2.5	2.8
18:1n–9	11.9	13.1	13.7	13.6	11.3	13.0
18:2n–6	43.7	17.3	4.7	1.6	42.5	17.6
20:5n–3	ND	8.7	12.3	13.9	ND	9.4
22:6n–3	ND	6.3	8.8	10.1	ND	5.6
SFA	43.7	39.0	37.7	36.4	45.6	40.7
MUFA	12.0	21.7	26.4	26.9	11.4	19.6
PUFA	44.3	39.3	35.9	36.7	43.1	39.7
PUFA/SFA	1.01	1.01	0.96	1.01	0.95	0.98
n–3 PUFA	0.3	19.4	27.2	30.9	0.3	19.1
n–6 PUFA	43.7	18.8	6.9	3.9	42.5	19.0
n–3/n–6	0.01	1.03	3.96	7.84	0.01	1.00

a: Values are percentages, except for ratios. See Table 1 footnote for definition of abbreviations.

b: Mixing proportions of the oils were as follows (wt%): in Experiment 1, 42.3:57.7 CCO-SO (Group I), 22.0:18.4:59.6 CCO-SO-FO (Group II), 14.6:4.1:81.3 CCO-SO-FO (Group III), and 12.6:0.3:87.1 CCO-SO-FO (Group IV); in Experiment 2, 45.3:54.7 CCO-SO (Group A) and 20.3:18.7:61.0 CCO-SO-FO (Group B).

Analysis

Experiment 1: Ninety-six rats were divided into the four dietary groups indicated in Table 2. The n–3/n–6 ratios of individual test lipids were adjusted to 0.01 (Diet I), 1.03 (Diet II), 3.96 (Diet III), and 7.84 (Diet IV). Sixty-six days after DMBA was administered, the rats were anesthetized with pentobarbital sodium (Somnopentyl, Kyoritsu, Tokyo, Japan). Blood was collected from the abdominal aorta. Tumors were removed rapidly, frozen in liquid N_2, and stored until use in the prostaglandin E_2 (PGE_2) assay. PGE_2 in tumors was extracted using a modification of the method of Fritshe and Johnston (24). Briefly, portions of the mammary tumors were quickly homogenized in phosphate-buffered saline using a Teflon-Potter homogenizer under liquid N_2. The homogenate was filtered through three layers of cheesecloth and then incubated at 37°C for one hour. Immediately after incubation,

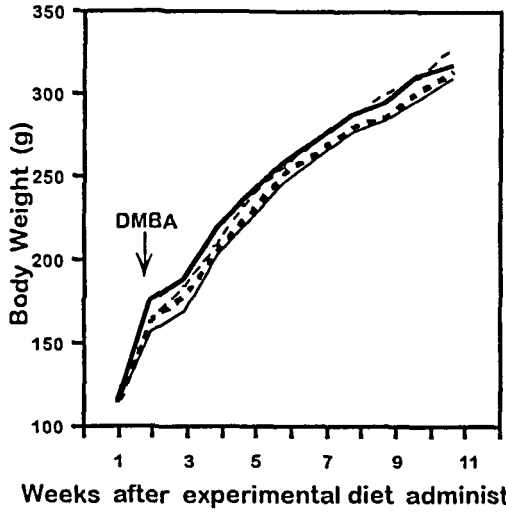

Figure 1. Body weight changes in 7,12-dimethylbenz[*a*]anthracene (DMBA)-treated rats. Thin solid line, Group I (n–3/n–6 = 0.01); thin dashed line, Group II (n–3/n–6 = 1.03); thick dashed line, Group III (n–3/n–6 = 3.96); thick solid line, Group IV (n–3/n–6 = 7.84).

aliquots of the homogenate were frozen. These samples were then stored at −80°C until analysis. PGE_2 concentration was determined by enzyme-linked immunosorbent assay (Amersham, Tokyo, Japan).

Experiment 2: Twenty-four rats were divided into the two dietary groups indicated in Table 2. The n–3/n–6 ratios of individual test lipids were adjusted to 0.01 (Diet A) and 1.00 (Diet B). Blood was collected in the same manner as in Experiment 1. An aliquot of blood was heparinized rapidly. Tumors and livers were removed rapidly, frozen in liquid N_2, and stored until use in the lipid peroxide assay.

Peripheral blood lymphocytes (PBLs) were isolated from heparinized blood using Percoll (Sigma Chemical) gradient centrifugation. Cell numbers and viability were determined by trypan blue exclusion as detected microscopically. PBLs were stained with fluorescein isothiocyanate-conjugated monoclonal antibody to rat CD4 (W3/25, Serotec, Kidlington, Oxford, UK) and phycoerythrin-conjugated monoclonal antibody to rat CD8 (MRC OX-8, Serotec) for two-color staining. Stained cells were analyzed using EPICs Elite (Coulter, Miami, FL) after the exclusion of dead cells by forward and side light scattering. Tumors and livers were homogenized in 1.15% (wt/vol) KCl buffer using an ice-cold Teflon-Potter homogenizer. Lipid peroxide concentration was determined by the method of Kikugawa and co-workers (22).

Table 3. Weights of Liver and Spleen in DMBA-Treated Rats[a–c]

Groups	Liver, g/100 g body wt	Spleen, mg/100 g body wt
I	3.18 ± 0.07	222 ± 21*
II	3.36 ± 0.21	309 ± 60†
III	3.25 ± 0.10	245 ± 25*,†
IV	3.35 ± 0.09	266 ± 18†

a: Values are means ± SE. DMBA, 7,12-dimethylbenz[*a*]anthracene.
b: Groups sharing a symbol (*,†) or without symbols are not significantly different ($p > 0.05$, analysis of variance combined with Duncan's new multiple comparison test).
c: n–3/n–6 ratios of dietary fats are as follows: 0.01 (Group I), 1.03 (Group II), 3.96 (Group III), and 7.84 (Group IV).

Statistical Analysis

In Experiment 1, intergroup differences were analyzed by analysis of variance and by Duncan's new multiple range test using the SAS system. The regression analysis was performed on Stat View statistical software (Cricket Software, Philadelphia, PA). In Experiment 2, intergroup differences were analyzed by Mann-Whitney's *U* test using Stat View.

Results

Experiment 1

Figure 1 shows the body weight gain of the rats in each dietary group. No significant difference was observed among the four groups. Table 3 shows weights of liver and spleen per 100 g of body weight. No significant differences in the liver weights were observed among the four dietary groups. The spleen weight was lower in the Diet I group than in the Diet II and IV groups. The reason for this differential observation is not clear.

Table 4 and Figures 2 and 3 show the effects of the n–3/n–6 ratio in dietary fat on the incidence, number, and weight of mammary tumors in rats. The incidence of mammary tumors tended to be lower in the Diet I group than in the other three groups; however, the time at which mammary tumors first appeared was similar in all the groups. The total number and weight of the mammary tumors in each group increased in a stepwise manner with increasing n–3/n–6 ratio. In particular, the weight of mammary tumors per tumor-bearing rat was higher in the Diet IV group than in the other groups. In addition, the result of regression analysis indi-

Table 4. Mammary Tumors in DMBA-Treated Rats[a,b]

Groups	Tumor Incidence at Necropsy, %	Latent Period, days	Total No. of Tumors	No. of Tumors/ Tumor-Bearing Rat	Total Tumor Wt/Group, g	Tumor Wt/ Tumor-Bearing Rat, g	Wt of 1 Primary Tumor, g
I	70	45 ± 1.5	108	6 ± 1.3	81	4.8 ± 1.3	0.8 ± 0.2
II	83	47 ± 1.8	120	6 ± 1.1	147	7.3 ± 2.2	1.3 ± 0.4
III	83	48 ± 1.9	134	7 ± 1.3	140	7.0 ± 1.9	0.8 ± 0.1
IV	88	46 ± 1.5	163	7 ± 1.3	227	11.8 ± 2.9	1.2 ± 0.2

a: Values are means ± SE.
b: n–3/n–6 ratios of dietary fats as in Table 3 footnote.

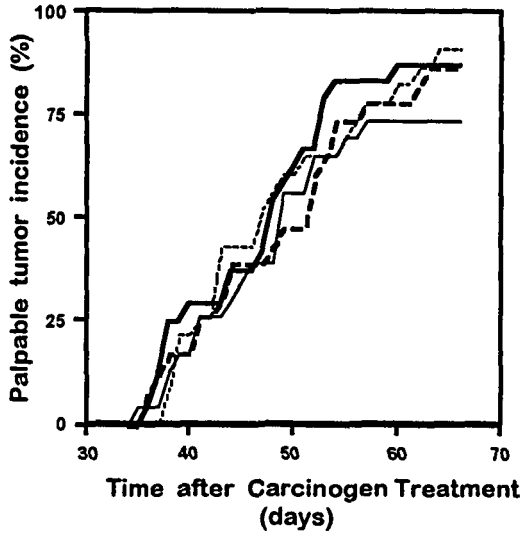

Figure 2. Cumulative palpable tumor incidence in DMBA-treated rats. Lines as defined in Figure 1 legend.

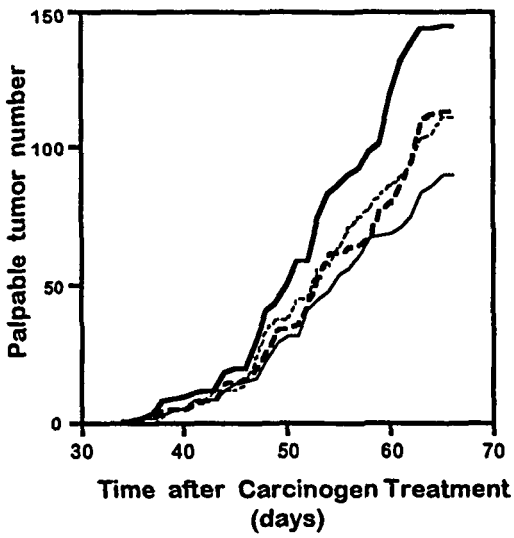

Figure 3. Cumulative palpable tumor number in DMBA-treated rats. Lines as defined in Figure 1 legend.

cated that the weights of mammary tumors per tumor-bearing rat were positively correlated with increasing n–3/n–6 ratio ($y = 1.278 \times x - 6.35$, $r^2 = 0.83$).

The PGE_2 concentration per gram of the mammary tumors was markedly lower in the rats in the Diet II group than in the rats in the Diet I group (Figure 4). However, the

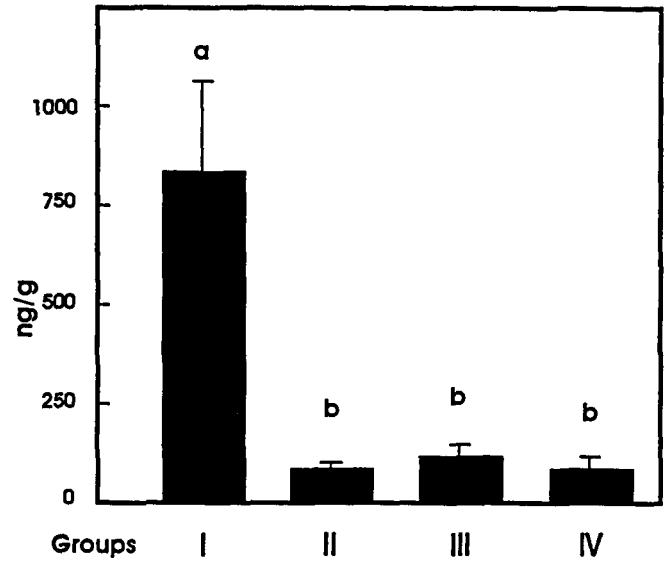

Figure 4. Prostaglandin E_2 (PGE_2) concentration in tumors of DMBA-treated rats. PGE_2 concentrations were determined by enzyme-linked immunosorbent assay. Groups sharing a letter (a, b) or without letters are not significantly different ($p > 0.05$, analysis of variance combined with Duncan's new multiple comparison test). n–3/n–6 ratios of dietary fats are as follows: 0.01 (Group I), 1.03 (Group II), 3.96 (Group III), and 7.84 (Group IV).

PGE_2 concentration was almost constant when the n–3/n–6 ratio was >1.03.

Experiment 2

The n–3/n–6 ratio was observed to have the same effect as in Experiment 1 on the incidence of mammary tumors and on the body weight gain of the rats (data not shown).

Although the n–3/n–6 ratio in the dietary fat was different in the two dietary groups, the number of PBLs was similar. The percentage of $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$ cells in the PBLs of the two groups was also similar (Table 5).

The concentration of lipid peroxides per liver weight in the rats was higher in the Diet B group than in the Diet A group, but no difference was observed in the concentration of lipid peroxides per weight of mammary tumors (Figure 5).

Discussion

In cases where FO-based diets were administered, suppression of mammary tumor incidence was indicated to result

Table 5. Number of PBLs and Flow Cytometry Analysis in DMBA-Treated Rats[a–c]

Groups	No. of PBLs, 10^6 cells/ml	Flow Cytometry Analysis, %			
		CD4SP	CD8SP	DN	CD4SP/CD8SP
A	1.3 ± 0.2	43 ± 4	22 ± 4	35 ± 7	2.3 ± 0.3
B	1.8 ± 0.2	45 ± 3	27 ± 3	27 ± 5	1.8 ± 0.1

a: Values are means ± SE.
b: Abbreviations are as follows: PBLs, peripheral blood lymphocytes; SP, single positive; DN, double negative.
c: n–3/n–6 ratios of dietary fats are as follows: 0.01 (Group A) and 1.00 (Group B).

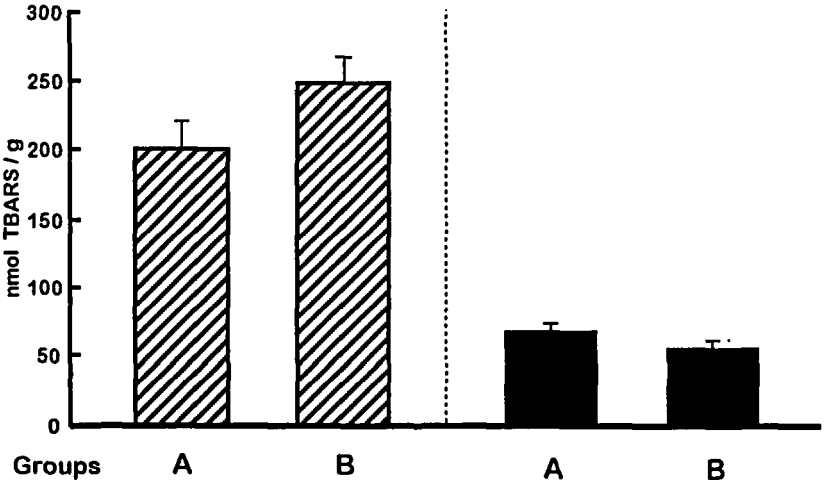

Figure 5. Lipid peroxide concentration of liver (left) and tumor (right) in DMBA-treated rats. Lipid peroxides were analyzed by thiobarbituric acid-reactive substances assay. Values are means ± SE. n–3/n–6 ratios of dietary fats are as follows: 0.01 (Group A) and 1.00 (Group B).

from the intake of n–3 PUFA. Cave and Jurkowski (17) showed in their study, in which a mixture of FO and CO that comprised 20% of the total feed weight was administered, that the latency was longer in a group fed fat with an n–3/n–6 ratio of 1.4 than in a group fed CO. Bunce and colleagues (18) observed a suppression of the incidence of mammary tumors in a group fed fat with an n–3/n–6 ratio of 1.18 compared with CO. Ip and associates (19) also observed suppression of the incidence of mammary tumors in a group fed fat with an n–3/n–6 ratio of 0.71 when dietary fat comprised 20% of the total feed weight similar to the above studies (17,18). In addition, in another study by Karmali and others (20), in which dietary fat comprised 23.5% of the total feed weight, suppression of mammary tumor incidence was observed in a group fed fat with an n–3/n–6 ratio of 0.91.

In the current study, using FO, SO, and CCO, we prepared various fat mixtures with constant P/S ratio and various n–3/n–6 ratios and fed the mixtures to rats as 10% of their total feed weight. The results indicate that the incidence of mammary tumors was not suppressed but that the total number and weight of the tumors were increased as a result of feeding the rats dietary fat with a high n–3/n–6 ratio. These results disagree with the results of previous studies (17–20).

Previously, Cohen and co-workers (21) reported that the n–3/n–6 ratio had selective inhibitory effects that were observed only when equal parts of FO and CO were fed to rats exhibiting *N*-nitroso-*N*-methylurea-induced mammary tumors. However, direct comparison of their results with ours is difficult, because 1) the total level of dietary fat they used (23.52%) is not equal to the level we used and may be too high for rats, since the level recommended by the American Institute of Nutrition is 5% of the total feed weight; 2) the P/S ratios of the dietary fat in the study of Cohen and co-workers varied from 2.0 to 4.7; and 3) we did not study the n–3/n–6 ratio of 0.5 with which they found a selective effect.

Many studies (18,25–28) regarding the relationship between eicosanoids and the incidence of mammary tumors have been carried out; however, the results are inconsistent. Some investigators reported a close relationship between the proliferation of tumor cells and the concentration of PGE_2, which is responsible for the suppressive effects of n–3 PUFA on mammary cancer. However, the other researchers denied any role for PGE_2 in the development of mammary cancer.

The analysis of PGE_2 concentration indicates the absence of a correlation between the PGE_2 concentration and the incidence of mammary tumors. This result supports the results of Carter and co-workers (29) and Fritsche and Johnston (24). Unfortunately, the leukotriene concentration was not determined in the current study, and therefore a complete evaluation of eicosanoid metabolism was impossible. However, in a previous study in which dietary fat with an n–3/n–6 ratio of 1.18 was used, which is similar to that used in our study, although the P/S ratios were different, a decrease in the leukotriene concentration was observed (18). Accordingly, the relationship between eicosanoid metabolism and the incidence of mammary tumors is assumed to be weak.

However, in Experiment 1 of the current study, although the 18:2 level in the diet fed to the rats in the Diet IV group was 1.8%, the incidence of mammary tumors was not suppressed but was promoted. According to the above-mentioned reports (9,17–20), this 18:2 level should be sufficiently low to suppress the incidence of mammary tumors. Considering the suppression of the incidence of mammary tumors equal to FO-fed groups observed in the CCO-fed groups by Craig-Schmidt and colleagues (10) and the current results, the suppression of the incidence of mammary tumors may depend not only on reducing the 18:2 level in dietary fat but also on the SFA content in the dietary fat; since in the current experiment the P/S ratio of fat fed to rats in all the groups was set at 1.0, the effect of suppression of mammary carcinogenesis induced by n–6 PUFA might possibly be masked by the effect of SFA. The fact that we did not observe any relationship between PGE_2 concentrations in tumor tissues and the incidence of mammary tumors may support this possibility.

Previously, Gonzalez and associates (15) reported that proliferation of tumor cells was suppressed by increased levels of lipid peroxide reactions (TBARS) in the tumors induced by FO administration. In Experiment 2 of the current study, although the concentration of lipid peroxides in the livers of rats was higher in the Diet A group than in the Diet B group, increases in the amounts of lipid peroxides in mammary tumor tissues were not observed. This fact may support a relationship between an increasing n–3/n–6 ratio and the increasing weight of mammary tumors observed in the rats in Experiment 1, which was contradictory to the results of previous reports. In contrast, Bertoli and others (30) reported recently that four weeks of low-dose administration of n–3 PUFAs that have the same ratio as our condition (n–3/n–6 ratio = 1.20) at lower quantity (as 5.5% of total feed weight) does not induce harmful modifications of oxidative cell metabolism. This apparent conflict between our results and their results may be explained by differences in quantity of dietary fats and/or the period of administration. However, these current results cannot be explained in terms of the P/S ratio or the n–3/n–6 ratio alone. In addition, the TBAR assay, which was used in Experiment 2 and the previous study (15), is a nonspecific method. Therefore, it is difficult to explain the effect of high n–3/n–6 ratio on tumor incidence by the results shown in Figure 5 alone.

In addition, in Experiment 2, to investigate whether an unbalanced immunosurveillance system is involved in the development of tumors observed in rats fed an n–3 PUFA-rich diet, we analyzed the effect of dietary fat on PBLs. The results indicate no effect of the n–3/n–6 ratio on the percentage of T lymphocyte subsets in PBLs. Without administration of DMBA, however, the activity of natural killer cells in PBLs in the group fed Diet A was significantly ($p < 0.05$) reduced compared with the group fed Diet B (unpublished data; the mean specific cytotoxicities were 15.3% and 9.0%, respectively). Despite the results obtained when DMBA was not administered, the decrease in activity of natural killer cells may explain the increase in the incidence of tumor development.

Nevertheless, further investigation is required not only of the response of organisms to lower n–3/n–6 ratios, because those used in the current study were higher than those in actual human diets, but also of the cause of the promotional effect of a diet with a high n–3/n–6 ratio on tumor development.

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