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Inhibition of 7,12-Dimethylbenz(a)anthracene-Induced Mammary Tumorigenesis by α-Linolenic Acid and Docosahexanoic Acid in Sprague-Dawley Rats

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Abstract: The effects of dietary ω-3 polyunsaturated fatty acid (ω-3 PUFA) on the development of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors were studied in female Sprague-Dawley rats. Animals were maintained on a test diet containing corn oil [CO; linoleic acid (18:2 ω-6), 48.9%], perilla oil [PO; α-linolenic acid (18:3 ω-3), 54.1%] or fish oil [FO; docosahexanoic acid (22:6 ω-3), 40.1%] as fat (12% w/w each) for 60 days starting one week after DMBA was administered (10 mg/rat, po). The first tumor in both the PO and FO groups appeared 18 days later than that in the CO group. The mean latent period for tumorigenesis was significantly longer in the PO than in the CO group. As compared with rats in the CO group, fewer rats in the other groups developed tumors, those which did develop tumors had fewer tumors, and those tumors which did develop were smaller. These observations were consistent with the findings at autopsy. Linoleic acid content in the microsomes from tumor cells was significantly lower in both the PO and FO groups than in the CO group. However, the microsomes from the PO and FO groups had a markedly high content of α-linolenic acid and docosahexanoic acid, respectively. These findings suggest that the dietary ω-3 PUFAs, α-linolenic acid and docosahexanoic acid, inhibit the development of DMBA-induced mammary tumors by changing the fatty acid composition of biological membranes.

Key words: Mammary tumor, 7,12-Dimethylbenz(a)anthracene (DMBA), α-Linolenic acid, Docosahexanoic acid

Epidemiological studies have revealed that a high-fat diet increases the incidence of breast cancer[1–5]. Several animal studies have shown that linoleic acid (LA; 18:2 ω-6), an essential fatty acid, is involved in this phenomenon[6–8], and that there is a dose-response relationship between the intake of LA and the development of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors[9].

In contrast, the fatty acids in fish oil are suggested to inhibit the development of mammary cancer. According to epidemiological studies, the mortality rate from breast cancer among female Greenland Eskimos, for whom marine products are a staple food, is lower than that among Western females[10][11]. ω-3 Type polyunsaturated fatty acids (ω-3 PUFA) including eicosapentaenoic acid (EPA; 20:5 ω-3), docosapentaenoic acid (DPA; 20:5 ω-3) and docosahexanoic acid (DHA; 22:6 ω-3) are

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; DMBA, 7,12-dimethylbenz(a)anthracene; EPA, eicosapentaenoic acid; LA, linoleic acid; α-LA, α-linolenic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.
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abundant in fish oil.

Using menhaden oil, Jurkowski and Cave[12] studied the effects of ω-3 PUFA on the development of N-methyl-N-nitrosourea-induced mammary tumors in rats and found that a diet containing 20% (w/w) menhaden oil inhibited tumorigenesis. Braden and Carroll[13] reported similar findings for DMBA-induced mammary tumors in rats. Menhaden oil also inhibited the growth of mammary cancer cells transplanted into rats and mice[14][15]. Since EPA accounts for about 29% of the fatty acids in menhaden oil, research attention is now being focused on the inhibitory effects of EPA on the development of breast cancer. However, there are few experimental reports of the use of ω-3 PUFA other than EPA as inhibitors of experimental mammary tumorigenesis.

In the present study, we compared the effects of perilla oil (PO), which is primarily composed of α-linolenic acid (α-LA; 18:3 ω-3), fish oil (FO), which is primarily composed of DHA, and corn oil, which is primarily composed of LA, on the development of DMBA-induced mammary tumors.

Materials and Methods

Animals and diets

Thirty-five-day-old female Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Shizuoka) were individually housed in stainless steel wire-bottomed cages in an air-conditioned room (22±2°C) with a 12-hour light (6 am to 6 pm) and dark cycle. They were allowed free access to a commerical pellet diet (Clea CE-2, Nippon Clea, Tokyo) and water.

Starting on day 42 after birth, their food was changed to a semipurified powder diet (basal diet) prepared by modifying the diet described by Lieber and DeCarli[16]. Dextrin-maltose was replaced with sucrose, and ethyl linoleate was substituted for safflower oil. Protein, fat and carbohydrate accounted for 15%, 25% and 60% of the total calories, respectively. The basal diet contained 16.0 g casein, 11.9 g corn and olive oils (1:3.3), 64.2 g sucrose, 0.2 g L-cystine, 0.1 g DL-methionine, 0.2 g choline bitartrate, 0.04 g vitamin mixture, 3.0 g mineral mixture and 4.3 g fiber (KC-flock, San-yo Kokusaku Pulp, Tokyo) per 100 g.

A single dose of 10 mg of DMBA (Tokyo Kasei, Tokyo) dissolved in 1 m*l* of corn oil was given to each rat on day 49 after birth. One week later, the animals were divided into 3 groups (10 animals each) and were given test diets containing corn oil (CO group), perilla oil (PO group) or fish oil (bonito oil:corn oil = 9:1; FO group) in place of the total fat content (12% w/w) of the basal diet. Each test diet was prepared daily by adding the appropriate oil to a stock powder, and rats were fed between 3 pm and 10 am. The stock diet and oils were stored separately in a cold room (4°C) until use. The oils were stored in dark bottles sealed under nitrogen. The fatty acid compositions of the oils are shown in Table 1. The CO was purchased from Ajinomoto Co. (Tokyo), and the PO and bonito oil were provided by Tsukuba Research Laboratory, Nippon Oil & Fats Co. (Tsukuba).

Rats were palpated twice weekly to monitor tumor development. To determine the tumor size, the two largest diameters of each tumor were measured with calipers and their mean

Table 1. Fatty acid composition of dietary fats[a]

Fatty Acid	Corn Oil	Perilla Oil	Fish Oil[b]
16:0	10.1	6.5	14.4
16:1	ND[c]	ND	4.1
18:0	1.6	2.1	2.3
18:1	31.4	17.3	11.9
18:2 ω-6	56.3	14.5	5.6
18:3 ω-3	0.4	54.1	0.1
20:1	ND	0.7	ND
20:5 ω-3	ND	ND	7.0
22:1	ND	3.3	ND
22:6 ω-3	ND	ND	40.1
Others	0.2	1.5	14.6

[a] The values represent fatty acid percentages (w/w).
[b] Fish oil = bonito oil + corn oil (9 : 1).
[c] ND, not detectable.

value was recorded. Body weights and food intake were also monitored twice a week.

Sixty days after DMBA was given, rats were anesthetized with ether and killed. The mammary tumors, liver, kidneys and spleen were excised and weighed. A portion of the periovary adipose tissue was collected to examine the fatty acid composition of body fat. This tissue was stored at −80°C until it was used.

The experiments were performed in accordance with the Guidelines for Animal Experiments, Medical University of Yamanashi.

Analysis of fatty acids

The tumors were homogenized in 1.15% KCl-0.01 M phosphate buffer (pH 7.4), using a Teflon-glass homogenizer. The homogenate was centrifuged at 10,000 ×g for 10 min at 0°C. The supernatant was then centrifuged at 105,000 ×g for 60 min at 4°C to obtain microsomes, which were stored at −80°C until they were used.

Lipids in the periovary adipose tissue and in the microsomes from the mammary tumors were extracted according to the method of Folch *et al.*'s method[17]. The fatty acids in the extract were methyl-esterified with boron fluoride-methanol[18]. The methyl esters of fatty acids were determined with a gas chromatograph equipped with a flame ionization detector (Hewlett-Packard Model 15890A, Sunnyvale, CA). Gas chromatography, with a 30 m × 0.25 mm fused silica capillary column (Carbowax 20 M, J & W Scientific, Folsom, CA) and helium as a carrier gas, was carried out at a split ratio of 100:1. The column temperature was 210°C. Chromatograms were analyzed with an integrator (Hewlett-Packard Model 3390A).

Statistics

Inter-group differences were analyzed by ANOVA, followed by the Student-Newman-Keuls' test[19]. The 0.05 level of probability was the criterion of significance.

Results

Food intake, body weight and weights of liver, kidneys and spleen

Figure 1 shows changes in food intake and body weight during the period in which the rats were maintained on the test diets. Animals in the FO group ate significantly less food than did the other groups when they were first changed from the basal diet to the test diets. On the third day after the diets were changed, the FO group animals were still eating significantly less than the CO group animals. Subsequently, however, there were no differences in food intake among the three groups. The average food intake during the period on the test diets was similar for the 3 groups: 12.1±0.6 g/day in the CO group, 12.5±0.8 g in the PO group and 11.6±1.3 g in the FO group.

At autopsy, performed 60 days after DMBA was given, animals in the FO group weighed significantly less than those in the PO group (Table 2). The mean liver weight in the FO group was significantly higher than that in the CO group. The weight of the spleen was also significantly higher in the FO group than in the other groups. There was no difference among the 3 groups with respect to kidney weight. The ratios of the weights of liver, spleen, and kidneys to body weight in the FO group were significantly higher than these ratios in the other groups ($p<0.05$).

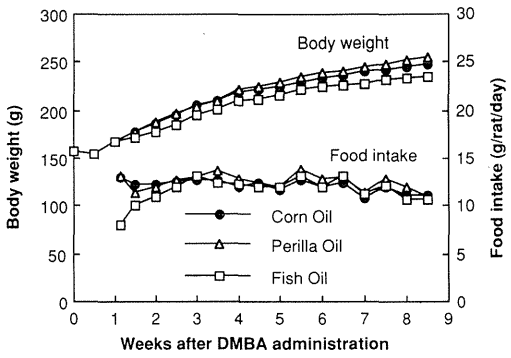

Fig. 1. Body weight and food intake during the experiment.

Tumorigenesis

Figure 2 shows the tumor incidence (A), the number of tumors (B) and the size of tumors (C), as determined by palpation. In the CO group, the first tumor appeared 30 days after DMBA was given, while in the other groups the first tumor appeared 48 days after DMBA was given (Fig. 2A). Throughout the experiment, the tumor incidence in the PO and FO groups was lower than that in the CO group.

The number of tumors developed was markedly higher in the CO group than in the other groups (Fig. 2B), and the tumors which did develop in the PO and FO groups were markedly smaller than those in the CO group (Fig. 2C). Eight weeks after DMBA was given,

Table 2. Body, liver, spleen, and kidney weights of rats fed different types of fats, measured at autopsy

Diet[d]	Weight (g)			
	Body	Liver	Spleen	Kidney
CO	247±17[a]	10.7±1.1	0.48±0.07	1.94±0.18
PO	255±24	11.5±1.5	0.52±0.07	2.03±0.18
FO	234±19[c]	12.6±1.8[b]	0.61±0.07[b,c]	2.04±0.20

[a] Values are the means±SD of 10 rats.
[b] Significantly different from the CO group, $p<0.05$.
[c] Significantly different from the PO group, $p<0.05$.
[d] CO, corn oil diet; PO, perilla oil diet; FO, fish oil diet.

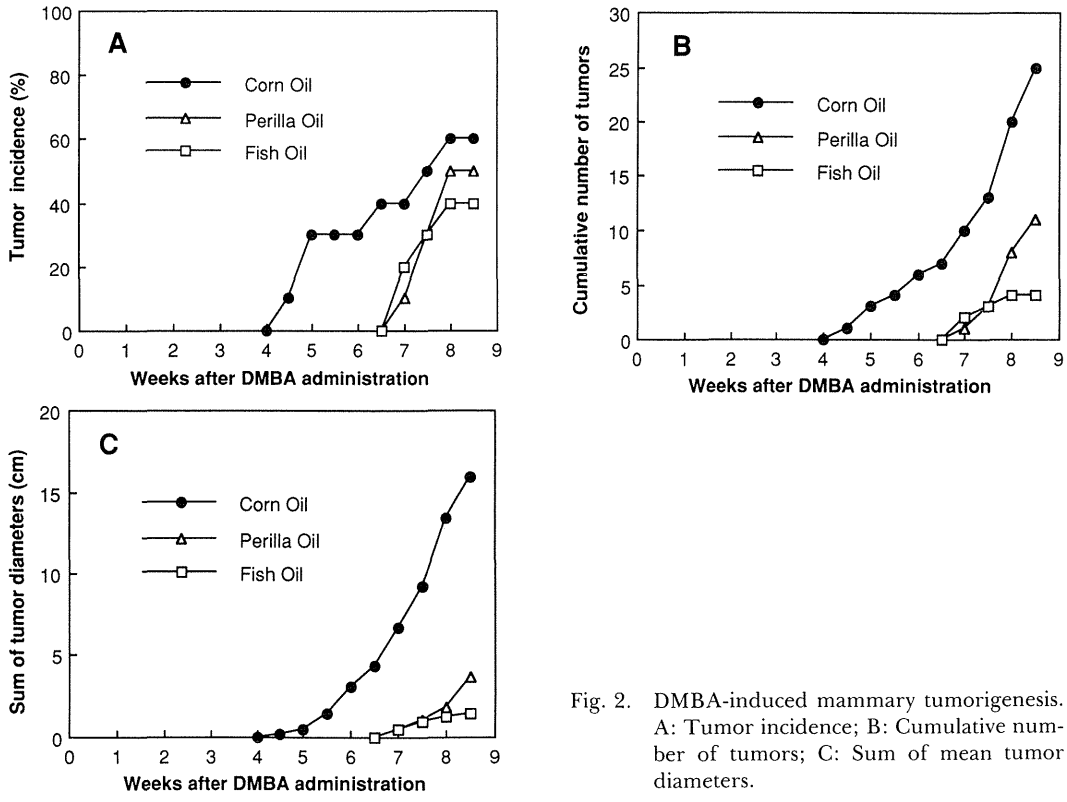

Fig. 2. DMBA-induced mammary tumorigenesis. A: Tumor incidence; B: Cumulative number of tumors; C: Sum of mean tumor diameters.

the FO group had fewer and smaller tumors than the PO group. The average number of days before the first appearance of tumor was 49.3±8.6 in the CO group, 56.4±3.2 in the PO group and 50.5±3.3 in the FO group. Thus, the PO group took longer to develop mammary tumors than did the CO group ($p<0.05$).

At autopsy, the tumor incidence was 70% in the CO and PO groups and 40% in the FO group, but this difference was not significant (Table 3). However, the groups could be ranked by the number of tumors as follows: CO group > PO group > FO group. The total weight of tumors was highest in the CO group: the values in the PO and FO groups were 1/5 and 1/20, respectively, of that in the CO group. While there was no significant difference in the number of tumors per tumor-bearing rat among the 3 groups, the average tumor weight per tumor-bearing rat was significantly higher in the CO group than in the other groups.

Fatty acid composition of periovary adipose tissue

The total content of saturated fatty acids (SFA) in the periovary adipose tissue was significantly higher in the FO group than in the other groups (Table 4). However, the FO group had significantly lower monounsaturated fatty acid (MUFA) content than did the other groups, this difference being due to a difference in oleic acid (18:1) content.

In all 3 groups, the major ω-6 PUFA was LA. However, the PO and FO groups had less LA than the CO group (48% and 41%, respectively).

The ratio of ω-3 PUFA to the total fatty acid content was less than 1% in the CO group, whereas it was 25% in the PO group and 22% in the FO group. Of the ω-3 PUFA, the PO group had a markedly high proportion of α-LA, and the FO group had a high proportion of DHA. The ω-3/ω-6 ratios in the PO and FO groups were 48 and 45 times, respectively, as large as the ratio in the CO group. The 3 groups could be ranked by PUFA/SFA (P/S) ratio as follows: PO group > CO group > FO group. These findings indicate that body fat composition can easily be changed by dietary fat and that existing fatty acids can be replaced by the major fatty acids that are consumed daily.

Fatty acid composition of tumor microsomal membrane lipids

There were no significant differences in SFA content among the 3 groups (Table 5). The palmitoleic acid (16:1) content was significantly higher in the FO group than in the other groups, but there were no significant differences in the total content of MUFA.

Regarding ω-6 PUFA, in all 3 groups, LA content was highest, followed by arachidonic acid (AA). The PO and FO groups had relatively little LA, 66% and 56%, respectively, of the amount in the CO group. AA content was highest in the CO group, and was significantly different from the content in the FO group.

Total ω-3 PUFA content in the PO and FO groups was 3.9 and 4.6 times as high, respec-

Table 3. Mammary tumors at autopsy in DMBA-treated rats fed different types of fats

Diet[c)]	Tumor Incidence (%)	Total No. of Tumors	No. of Tumors/ Tumor-Bearing Rat	Total Tumor Weight (g)	Tumor Weight/ Tumor-Bearing rat (g)
CO	70	25	3.57±2.70[a)]	13.6	1.94±2.23
PO	70	12	1.71±0.76	2.9	0.41±0.33[b)]
FO	40	7	1.75±0.50	0.7	0.18±0.13[b)]

[a)] Values are the means±SD (CO, n=7; PO, n=7; FO, n=7).
[b)] Significantly different from the CO diet group, $p<0.05$.
[c)] CO, corn oil diet; PO, perilla oil diet; FO, fish oil diet.

tively, as that in the CO group. The PO group had a markedly higher α-LA content and the FO group had a markedly higher DHA content than the CO group.

Although there were no significant difference in the P/S ratio among the 3 groups, the ω-3/ω-6 ratios in the PO and FO groups were 6.1 and 8.6 times higher, respectively, than that in the CO group.

Discussion

The findings of the present study indicate that the ω-3 PUFAs, α-LA (PO) and DHA (FO), inhibit the development of DMBA-induced mammary tumors in rats, as compared with CO, which predominantly contains LA, an ω-6 PUFA. Several studies have reported the inhibitory effects of α-LA on the development of experimental tumors. Hori *et al.*[20] observed that PO suppressed the pul-

Table 4. Fatty acid composition of periovary adipose tissue in rats fed different types of fats

Fatty Acid	Corn Oil	Perilla Oil	Fish Oil
Saturated Fatty Acids (SFA)			
12:0	ND[a]	ND	ND
14:0	0.99±0.08[b]	1.10±0.06[c]	2.18±0.13[c,d]
16:0	21.3±0.6	20.4±0.5[c]	25.3±1.1[c,d]
18:0	2.80±0.16	3.08±0.17[c]	3.87±0.21[c,d]
Total	25.1±0.7	24.6±0.6	31.4±1.2[c,d]
Monounsaturated Fatty Acids (MUFA)			
16:1	3.42±0.29	4.44±0.47[c]	5.47±0.81[c,d]
18:1	33.2±0.6	28.7±0.5[c]	22.9±0.7[c,d]
20:1	0.35±0.02	0.31±0.02[c]	0.54±0.04[c,d]
Total	37.0±0.9	33.4±0.9	28.9±1.4[c,d]
ω-6 Polyunsaturated Fatty Acids (ω-6 PUFA)			
18:2	34.8±1.4	16.7±0.7[c]	14.2±2.6[c,d]
18:3	0.18±0.02	0.09±0.02[c]	0.09±0.03[c]
20:2	0.23±0.02	0.11±0.01[c]	0.13±0.02[c,d]
20:3	0.24±0.02	0.09±0.01[c]	0.18±0.02[c,d]
20:4	1.04±0.15	0.36±0.04[c]	1.18±0.08[c,d]
22:4	0.39±0.06	0.05±0.03[c]	0.14±0.09[c,d]
22:5	0.13±0.08	0.01±0.01[c]	1.38±0.16[c,d]
Total	37.0±1.4	17.4±0.7[c]	17.3±2.7[c]
ω-3 Polyunsaturated Fatty Acids (ω-3 PUFA)			
18:3	0.75±0.06	22.8±1.3[c]	0.51±0.10[c,d]
20:5	0.01±0.01	0.70±0.13[c]	1.20±0.16[c,d]
22:5	0.03±0.04	0.84±0.18[c]	1.14±0.18[c,d]
22:6	0.13±0.02	0.39±0.05[c]	19.6±2.2[c,d]
Total	0.92±0.08	24.7±1.5[c]	22.4±2.6[c,d]
ω-3/ω-6	0.03±0.01	1.43±0.14[c]	1.34±0.32[c]
P/S[e]	1.52±0.09	1.71±0.07[c]	1.27±0.12[c,d]

[a] ND, not detectable.
[b] Values are the means±SD (n=10). The values shown are percentages of the total fatty acid content.
[c] Significantly different from the corn oil group, $p<0.05$.
[d] Significantly different from the perilla oil group, $p<0.05$.
[e] P/S, ratio of polyunsaturated fatty acids to saturated fatty acids.

monary metastasis of Yoshida sarcoma cells injected intravenously in rats. Fritsche and Johnston[21] reported that linseed oil (α-LA, 56%) inhibited the pulmonary metastasis of murine mammary adenocarcinoma inoculated subcutaneously in BALB/c mice. Linseed oil was also reported to inhibit the growth of BN 472 tumors transplanted into subcutaneous tissue in rats[22]. Cameron *et al.*[23] found that a diet containing 6% linseed oil inhibited the development of DMBA-induced mammary tumors in mice (a diet containing 3% linseed oil was ineffective). These reports are consistent with the present finding that PO (α-LA, 51%) inhibits DMBA-induced tumorigenesis.

To our knowledge, the present study is the first to demonstrate that DHA, another ω-3 PUFA, inhibits the development of DMBA-induced mammary tumors. The present findings suggest that DHA (FO) inhibits tumor development (as reflected in the number and size of tumors) more strongly than does α-LA

Table 5. Fatty acid composition of tumor microsomal membrane lipids in rats fed different types of fats

Fatty Acid	Corn Oil	Perilla Oil	Fish Oil
Saturated Fatty Acids (SFA)			
12:0	4.61±2.64[a]	6.66±6.73	7.40±2.00
14:0	2.85±1.10	3.68±2.78	4.04±0.84
16:0	20.5±1.8	20.7±2.1	22.3±0.4
18:0	11.7±2.5	11.7±3.8	9.50±0.32
Total	39.6±3.0	42.7±7.1	43.2±3.4
Monounsaturated Fatty Acids (MUFA)			
16:1	2.04±0.33	2.36±0.52	3.37±0.47[c]
18:1	22.7±2.2	21.2±2.7	21.1±0.93
20:1	0.23±0.23	0.35±0.20	0.21±0.30
Total	25.0±2.5	23.9±3.5	24.7±1.3
ω-6 Polyunsaturated Fatty Acids (ω-6 PUFA)			
18:2	17.2±4.0	11.3±2.6[c]	9.66±0.60[c]
18:3	ND[b]	ND	ND
20:2	0.34±0.16	0.20±0.13	ND
20:3	0.52±0.32	0.56±0.35	0.39±0.32
20:4	10.5±3.6	7.76±4.83	5.61±0.64[c]
22:4	2.28±0.91	0.51±0.32[c]	0.24±0.17[c]
22:5	1.37±0.64	0.49±0.28[c]	1.39±0.24[d]
Total	32.2±2.5	20.9±4.7[c]	17.3±0.7[c]
ω-3 Polyunsaturated Fatty Acids (ω-3 PUFA)			
18:3	1.32±0.90	6.33±2.43[c]	1.46±0.41[d]
20:5	0.06±0.12	1.90±1.27	1.06±0.09[c]
22:5	0.31±0.45	2.60±1.62[c]	1.10±0.18[c][d]
22:6	1.55±0.53	1.69±0.51	11.2±1.2[c][d]
Total	3.23±1.62	12.5±2.4[c]	14.9±1.8[c]
ω-3/ω-6	0.10±0.05	0.61±0.07[c]	0.86±0.09[c][d]
P/S[e]	0.90±0.13	0.81±0.27	0.75±0.11

[a] Values are means±SD (Corn oil, n=7; Perilla oil, n=7; Fish oil, n=4). The values shown are percentages of the total fatty acid content.
[b] ND, not detectable.
[c] Significantly different from the corn oil group, $p<0.05$.
[d] Significantly different from the perilla oil group, $p<0.05$.
[e] P/S, ratio of polyunsaturated fatty acids to saturated fatty acids.

(PO) (Fig. 2B and Table 3). However, animals in the FO group, which consumed less food during the first week on the test diet than did animals in the PO group (Fig. 1), weighed significantly less at autopsy (Table 2). The development of DMBA-induced mammary tumors is known to be closely related to food intake: a reduction in food intake, particularly within one week before or after DMBA administration, has been shown to strongly inhibit the development of tumors[24]. It is possible, therefore, that the lower food intake in the FO group during this "critical period" affected the subsequent tumorigenesis.

The mechanism by which ω-3 PUFA inhibit the development of mammary tumors is unknown. Analysis of fatty acid composition revealed that the ω-6 PUFA in body fat and in cellular membrane lipids can easily be replaced by dietary ω-3 PUFA (Tables 4 and 5). DHA was little metabolized and served as a major component of the cell membrane in the tumors (Table 5). In contrast, α-LA was metabolized into EPA or into other ω-3 PUFA with longer chains.

Prostaglandin synthesized from AA seems to be involved in the growth and metastasis of tumors, through as yet unknown mechanisms[25–28]. EPA[14)29)], α-LA[30)31)], and DHA [32)] are known to competitively inhibit the biosynthesis of prostaglandin from AA. Therefore, the changes in the fatty acid composition of cell membranes observed in this study, may have affected the synthesis of prostaglandin from AA.

The development of DMBA-induced mammary tumors depends on prolactin and on ovarian hormones[33]. AA is known to modulate the binding of sex and steroid hormones, such as estrogen, progestin, androgen and glucocorticoid, to their receptors[34)35)]. Thus, it is also likely that changes in the fatty acid composition of cell membranes may affect DMBA tumorigenesis by modulating these hormone receptors.

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