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Suppression of Hepatic Lipogenic Enzyme by Dietary Fish Oil in Rat Hepatocarcinogenesis

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ABSTRACT : *This study was designed to examine the effects of polyunsaturated fatty acid (PUFA) from different sources on hepatic lipogenic enzyme and peroxisomal β-oxidation in murine hepatocarcinogenesis initiated by diethylnitrosamine (DEN). Male Sprague-Dawley rats were fed one of three diets containing 10% (w/w) fat; fish oil-corn oil blended (FO), corn oil-beef tallow-fish oil blended (CF), or corn oil-beef tallow-perilla oil blended (CP), from the gestation period. At 10 weeks, animals were received a single intraperitoneal injection of DEN (200 mg/kg body weight), were subjected to two-thirds partial hepatectomy 3 weeks later and were sacrificed 8 weeks after DEN initiation. The areas of placental glutathione S-transferase (GST-P) positive foci were significantly smaller in rats fed fish oil containing diets (FO and CF) than those fed CP diet. Fish oil feeding significantly decreased the activities of lipogenic enzyme. Rats fed fish oil containing diets (FO, CF) exhibited the lower fatty acid synthase (FAS) activity than those fed CP diet and FAS activity was positively correlated with the areas of GST-P positive foci. Glucose-6-phophate dehydrogenase activity was the lowest and peroxisomal β-oxidation was stimulated in rats fed FO diet compared to other groups. It was also found that serum cholesterol was decreased in FO group. Therefore, the preventive effect against hepatocarcinogenesis and hypolipidemic effect of fish oil can be explained partly by suppression of the hepatic lipogenesis and by increase of peroxisomal β-oxidation.*

Key Words : *n-3 Long chain polyunsaturated fatty acid, Hepatocarcinogenesis, Placental glutathione S-transferase (GST-P) positive foci, Lipogenesis, Peroxisomal β-oxidation*

I. INTRODUCTION

Dietary fat has been found to influence the development of cancer in both epidemiological studies in human (Prentice *et al.*, 1988) and experimental studies in laboratory animals (Glauert *et al.*, 1991). The effect of dietary fat on tumor promotion depends on the amount and type of fatty acids (Reddy and Sugie, 1988). High fat diet elevates the toxic effects of nuclear-damaging agents and carcinogens (Birt, 1990). Dietary soybean oil, corn oil and safflower oil, rich in n-6 polyunsaturated fatty acid (PUFA), promote tumor growth compared with beef tallow, but fish oil, rich in n-3 PUFA, has adverse effect (O'Connor *et al.*, 1989).

The mechanism of dietary fat modulation of carcinogenesis is not clearly understood, but a number of hypotheses have been proposed. One hypothesis proposes the involvement of free radicals and lipid peroxidation in cell damage (Tappel, 1979). Another one proposes that the type of dietary fatty acid might affect the lipid composition of membrane (Burns and Spector, 1990). Accordingly, hepatic microsomal drug-metabolizing enzymes as well as membrane-bound enzyme such as ATPase, protein kinase could also be modified (Stubbs and Smith, 1984).

Some early studies showed elevated levels of fatty acid synthesis in tumor tissues compared with in normal tissue (Medes *et al.*, 1953), without appreciating the significance of the observations. Recently, a few studies demonstrated that carcinomas of the breast, colon, prostate, and endome-

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LIST OF ABBREVIATION: AOX (acyl-CoA oxidase), DEN (diethylnitrosamine), DHA (docosahexaenoic acid, C22:6, n-3), EPA (eicosapentaenoic acid, C20:5, n-3), FAS (fatty acid synthase), G6PDH (glucose 6-phosphate dehydrogenase), GST-P (placental glutathione S-transferase), LNA (α-linolenic acid, C18:3, n-3), ME (malic enzyme), PUFA (polyunsaturated fatty acid)

trium express high levels of fatty acid synthase (FAS), the major enzyme of fatty acid biosynthesis (Alo *et al.*, 1996) and FAS inhibitor, cerulenin, was proved to be selectively cytotoxic to cell lines derived from human malignancies (Pizer *et al.*, 1996). In addition, several studies observed the marked increase in glucose-6-phosphate dehydrogenase activity in putative pre-neoplastic lesions in the liver, pancreas and a variety of different organs (Moore *et al.*, 1983).

Dietary fat has different effect on lipogenesis. Dietary PUFA are potent inhibitors of hepatic lipogenesis while saturated and monosaturated fatty acids exhibit little to no inhibitory capability (Odin *et al.*, 1987). Especially n-3 long chain PUFA are the most potent inhibitors of hepatic fatty acid and triglyceride synthesis. Thus, the possibility that the modulationg effect of n-3 PUFA on carcinogenesis is related to low lipogenic capacity could be proposed, but studies have been very limited.

The present study was designed to examine the effects of PUFA from different sources on hepatocarcinogenesis following long term feeding from the gestation period and to determine whether this effect is correlated with hepatic lipogenesis. As different sources of n-3 fatty acids perilla oil and fish oil were chosen, the former is a rich source of α-linolenic acid (LNA; 18 : 3, n-3) and the latter is major source of eicosapentaenoic acid (EPA; 20` : 5, n-3) and docosahexaenoic acid (DHA; 22 : 6, n-3).

II. MATERIALS AND METHODS

1. Animals and diet

Female Sprague-Dawley rats aged 10 weeks were divided into three groups and were mated. During the gestation period, the dams were fed on each of the three experimental diets containing 10% (w/w) of various fats (Table 1). As shown in Table 2, the composition of PUFA was the sole variable and polyunsaturated to saturated fatty acid (p/s) ratios were the same among the dietary groups. The FO diet was added 1.8% of corn oil to furnish 2~3% of essential fatty acid and the CP and CF diet groups differed sources of n-3 PUFA. The CP diet contained perilla oil and the CF diet included fish oil as n-3 PUFA source. And α-tocopherol was supplemented to contain 200 mg in 100 g oil. After weaning, the male pups were fed the same diet as their dams. The rats were maintained on a 12 h light and 12 h dark daily cycle, were given food and water *ad libitum*. Meals were offered daily and the diets were stored in the freezer (-20°C) and replenished N_2 gas between meals to minimize the opportunity for peroxidation of lipids. Hepatocellular chemical carcinogenesis was induced using the

Table 1. Composition of experimental diets (g/100 g diet)

Component/Diet	FO	CF	CP
Corn starch	59.7	59.7	59.7
Casein	20.0	20.0	20.0
α-Cellulose	5.0	5.0	5.0
Vitamin mixture[a]	1.0	1.0	1.0
Salt mixture[b]	4.0	4.0	4.0
DL-Methionine	0.3	0.3	0.3
Beef Tallow	-	3.5	4.0
Corn oil	1.8	5.0	5.0
Perilla oil	-	-	1.0
Fish oil	8.2	1.5	-
α-Tocopherol	0.019	0.015	0.015

[a]Nutritional Biochemicals. ICN Life Science Group. Cleveland, Ohio. Vitamin mixture is composed of; vit. A acetate (500,000 IU/g) 1.8 g, vit D conc. (850,000 IU/g) 0.125 g, α-tocopherol (250 IU/g) 22.0 g, ascorbic acid 45.0 g, inositol 5.9 g, choline chloride 75.0 g, menadione 2.25 g, p-aminobenzoic acid 5.0 g, niacin 4.25 g, riboflavin 1.0 g, pyridoxine hydrochloride 1.0 g, calcium pantothenic acid 3.0 g, biotin 0.02 g, folic acid 0.09 g, vit B_{12} 0.00135 g, and dextrose to 1 kg.
[b]Composition of salt mixture, g/kg mixture: $CaHPO_4$ 500 g, NaCl 74 g, K_2SO_4 52 g, potassium citrate monohydrate 220 g, MgO 24 g, manganase carbonate (43~48% Mn) 3.5 g, ferric citrate (16~17% Fe) 6.0 g, cupric carbonate (53~55% Cu) 0.3 g, KIO_3 0.01 g, chromium potassium sulfate 0.55 g, $Na_2SeO_3 \cdot 5H_2O$ 0.11 g, sucrose, finely powdered 118.0 g.

Table 2. Fatty acid composition of diets (%)

	FO	CF	CP
Saturated fatty acid	31.6	28.3	26.1
Monounsaturated fatty acid	25.8	37.6	37.9
Polyunsaturated fatty acid	42.5	33.3	34.8
n-6/n-3 ratio[a]	0.40	4.23	4.00
p/s ratio[b]	1.34	1.18	1.33

FO, diet containing a fat mixture composed of fish oil and corn oil (8.2:1.8); CF, diet containing a fat mixture composed of corn oil, beef tallow and fish oil (5:3.5:1.5); CP, diet containing a fat mixture composed of corn oil, beef tallow and perilla oil (5:4:1).
[a]Total n-6 fatty acids/total n-3 fatty acids in experimental diet.
[b]Total polyunsaturated fatty acids/total saturated fatty acids in experimental diet.

modified method of medium-term bioassay protocol (Ito *et al.*, 1992). Animals received a single intraperitoneal injection of diethylnitrosamine (DEN) (200 mg/kg body weight) dissolved in saline at aged 10 weeks, were subjected to two-thirds partial hepatectomy (PH) 3 weeks later and were sacrificed 8 weeks after DEN initiation.

2. Sampling and tissue preparation

Animals were decapitated after 12 h fasting. The blood was collected in tubes and centrifuged under cold condition at 1,500×g for 20 min to separate the plasma. Livers were immediately removed, finely minced in 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA, and then homogenized. The homogenate was centrifuged at 800×g for 10 min and the supernatant was used for the assay of peroxisomal β-oxidation, acyl-CoA oxidase and catalase. The remaining supernatant was centrifuged at 105,000×g for 1 h and the resulting supernatant was used for the assay of lipogenic enzyme.

3. Placental glutathione S-transferase (GST-P) positive foci

At autopsy, livers were excised and sections 2~3 mm thick were cut with a blade. These liver slices were fixed in ice cold acetone for immunohistochemical examination of GST-P positive foci. The avidine-biotin peroxidase complex method was used to demonstrate GST-P positive liver foci, a putative preneoplastic lesion. Immunohistochemical analysis was carried out with sequential treatments of rabbit anti rat placental glutathione S-transferase as a primary antibody, swine anti rabbit IgG antibody as a secondary antibody and peroxidase-antiperoxidase complex. Final visualization of GST-P positive foci was enzymatically activated by 3,3-diaminobenzidine and H_2O_2 as substrates. The areas of the GST-P positive foci >0.2 mm in diameter and total areas of liver sections examined were measured using an image analyzer.

4. Lipogenic enzyme assay

FAS activity was determined spectrophotometrically by the method of Linn (1981). Malic enzyme (ME) activity was measured using the method of Ochoa (1969) and G6PDH activity was assayed by the method of Löhr and Waller (1974).

5. Peroxisomal β-oxidation, acyl-CoA oxidase and catalase assay

Peroxisomal β-oxidation was determined according to Lazarow (1981), by measuring cyanide insensitive palmitoyl-CoA dependent NAD reduction. Acyl-CoA oxidase (AOX) activity was assayed by measuring the palmitoyl-CoA dependent H_2O_2 production by the method of Hashimoto *et al.* (1981). Catalase activity was determined by the method of Aebi (1984), measuring the enzymatic decomposition of H_2O_2.

6. Lipid analyses

Total hepatic lipid were extracted by the method of Folch *et al.* (1957). Triglycerides (TG) and total cholesterol contents in the liver extract and plasma were assayed by using commercial enzymatic kits (Youngdong Pharm. Korea).

7. Protein assay

Protein amounts were measured by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

8. Statistical analysis

All statistical analyses were carried out using ANOVA and Duncan's multiple range test, p value of <0.05 was selected as a limit of statistical significance. The statistical program used was SAS package.

III. RESULTS AND DISCUSSION

Various dietary fats did not influence food intakes and body weight gains during the experimental period. In addition, no significant difference in the final body weight was found.

Fig. 1 shows that GST-P positive foci development

was significantly different among dietary fats. The CF diet group had the greatest inhibitory effect and the FO diet group showed smaller area of GST-P positive foci than the CP diet group. Several types of GST forms are known to be elevated, but GST-P is the most effective marker for DEN-initiated lesions (Satoh *et al.*, 1989). Moreover, Ogiso *et al.* (1990) proved that the degree of induction of GST-P positive foci and nodules in the medium-term bioassay protocol for liver carcinogens directly corresponds with the incidence of hepatocellular carcinomas revealed in long-term *in vivo* systems. Consequently, the present result suggests that fish oil containing diets could inhibit liver tumor development. On the other hand, Choi *et al.* (1996) showed that the areas of GST-P positive foci at 8 weeks were not different among oil groups, but after long-term feeding of the experimental diets for 22 weeks, tuna oil markedly reduced the area. Therefore, feeding various fats in early stages of growth and development from the gestation period might potentiate the effect on hepatocarcinogenesis.

Lipogenic enzyme activities in the hepatic cytosol from rats fed experimental diets are shown in Table 3. The activities of G6PDH and FAS were suppressed in FO diet group. Similar results were observed in several studies (Toussant *et al.*, 1981; Suh *et al.*, 1990), indicating that n-3 PUFA, especially long chain PUFA (*e.g.*, EPA, DHA), are the most potent inhibitors of hepatic lipogenesis. But ME activity was not different among the dietary groups in this study.

The ribulose-5-phosphate generated by G6PDH might be necessary for nucleotide synthesis (Yoshimoto *et al.*, 1983), required in promotional stages of hepatic carcinogenesis. Therefore, this study suggests that feeding of FO diet decrease the generation of ribulose-5-phosphate, to have protective effect against hepatocarcinogenesis. Moore *et al.* (1986) observed marked increase in the activities of G6PDH and ME in putative preneoplastic lesions induced by N-ethyl-N-hydroxyethylnitrosamine. The generation of NADPH has been believed to enhance drug detoxifying potential. However, since the cytochrome P450 systems which might require NADPH for hydroxylating reactions are reduced in putative preneoplastic and neoplastic liver lesion (Becker and Stout, 1984), it is thought pertinent to examine the possibility that NADPH might be required for lipogenesis rather than drug metabolism. Therefore, the feeding of FO diet could result in decreased hepatic lipogenesis.

FAS is the rate-limiting enzyme for fatty acid synthesis in mammalian systems and synthesizes long chain, saturated fatty acids from acetyl-CoA, malonyl-CoA and NADPH (Wakil *et al.*, 1983). The

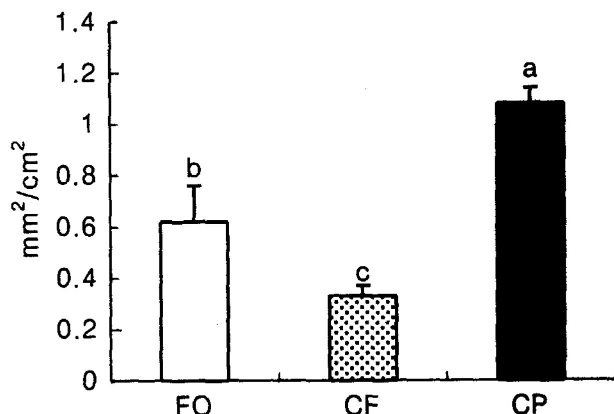

Fig. 1. Effect of dietary PUFA on the area of GST-P positive foci in rat hepatocarcinogenesis. FO, diet containing a fat mixture composed of fish oil and corn oil (8.2 : 1.8); CF, diet containing a fat mixture composed of corn oil, beef tallow and fish oil (5 : 3.5 : 1.5); CP, diet containing a fat mixture composed of corn oil, beef tallow and perilla oil (5 : 4 : 1). Values are mean±SE. Means with the same subscript are significantly different at p< 0.05 by Duncan's multiple range test.

Table 3. Effect of dietary PUFA on the hepatic lipogenic enzyme activities in rat hepatocarcinogenesis

Group	Number of rats	Fatty acid synthase (nmol NADPH oxidized/ min/mg protein)	Glucose-6-phosphate dehydrogenase (nmol NADP reduced/ min/mg protein)	Malic enzyme (μmol NADP reduced/ min/mg protein)
FO	5	2.77 ± 0.39^b	8.37 ± 0.56^b	11.25 ± 0.93^a
CF	5	4.22 ± 0.37^b	16.07 ± 2.78^a	10.23 ± 1.48^a
CP	6	6.41 ± 0.71^a	18.49 ± 1.55^a	13.75 ± 1.15^a

FO, diet containing a fat mixture composed of fish oil and corn oil (8.2 : 1.8)+carcinogen treatment (DEN+ partial hepatectomy); CF, diet containing a fat mixture composed of corn oil, beef tallow and fish oil (5 : 3.5 : 1.5)+carcinogen treatment (DEN+partial hepatectomy); CP, diet containing a fat mixture composed of corn oil, beef tallow and perilla oil (5 : 4 : 1)+carcinogen treatment (DEN+partial hepatectomy). Values are mean±SE. Means with the same subscript are significantly different at p<0.05 by Duncan's multiple range test.

enzyme is under complex nutritional and hormonal control, among them, the amount and type of dietary fat have regulatory ability. As shown in this present result, fish oil rich in n-3 long chain PUFA depressed FAS activity strongly. When compared the CF diet group with CP diet group, this effect of fish oil rich in EPA and DHA was stronger than that of perilla oil rich in LNA even small amount in reducing FAS activity. In addition, the activities of FAS were positively correlated with the areas of GST-P positive foci ($r=0.54891$, $p<0.05$). These findings suggest that fish oil may be preventive against hepatocarcinogenesis by suppressing the fatty acid synthesis. Recent studies have demonstrated that elevated fatty acid synthesis and high levels of FAS expression occur in carcinomas of the breast, endometrium, prostate, and colorectal neoplasia (Alo *et al.*, 1996; Rashid *et al.*, 1997). Furthermore, carcinoma cell lines derived from human malignancies were dependent upon endogenous fatty acid synthesis for growth (Pizer *et al.*, 1996) and FAS inhibitor, cerulenin, is selectively cytotoxic to carcinoma cell lines. Thus, suppression of FAS may be useful treating cancer *in vivo*.

Compared with other diet groups, FO diet group increased peroxisomal-β-oxidation, the specific activity of peroxisomal AOX and catalase significantly (Table 4). Similar results were observed in other studies (Yamazaki *et al.*, 1987). The stimulation of peroxisomal β-oxidation activity can be interpreted to have both sides. The increase of peroxisomal β-oxidation seems likely to lead the oxidation of fatty acid, thus reducing the fatty acid substrate *in vivo*. On the other hand, the proliferation of peroxisome might cause the formation of H_2O_2 and other reactive oxygen species, thus increasing the lipid peroxidation (Kvannes *et al.*, 1995). Lipid peroxidation decreased the microsomal membrane integrity and was associated with the promotion of carcinogenesis (Slaga *et al.*, 1981). Several studies reported that lipid peroxidation was enhanced by fish oil (Halminski *et al.*, 1991). Consequently, the enhanced lipid peroxidation, in spite of the increased catalase activity, might explain partly the result that FO diet with much higher n-3 PUFA had the larger area of the GST-P positive foci than CF diet with less fish oil.

Table 4. Effect of dietary PUFA on the peroxisomal β-oxidation, acyl-CoA Oxidase and catalase activities in rat hepatocarcinogenesis

Group	Number of rats	Peroxisomal β-oxidation (nmol NAD reduced/ min/mg protein)	Acyl-CoA oxidase (nmol H_2O_2 produced/ min/mg protein)	Catalase (μmol H_2O_2 decomposed/ min/mg protein)
FO	5	6.40 ± 0.29^a	5.31 ± 0.40^a	69.21 ± 2.45^a
CF	5	4.08 ± 0.52^b	2.68 ± 0.20^b	56.71 ± 4.12^b
CP	6	3.35 ± 0.33^b	1.51 ± 0.13^c	51.79 ± 1.66^b

FO, diet containing a fat mixture composed of fish oil and corn oil (8.2:1.8)+carcinogen treatment (DEN+partial hepatectomy); CF, diet containing a fat mixture composed of corn oil, beef tallow and fish oil (5 : 3.5 : 1.5)+ carcinogen treatment (DEN+partial hepatectomy); CP, diet containing a fat mixture composed of corn oil, beef tallow and perilla oil (5 : 4 : 1)+carcinogen treatment (DEN+ partial hepatectomy). Values are mean±SE. Means with the same subscript are significantly different at $p<0.05$ by Duncan's multiple range test.

Table 5 shows the effect of dietary fat on the level of serum lipid and the liver lipid. The total liver lipid and liver TG were not different among the diet groups. The effect of fish oil on various lipid level in liver tissue has been inconsistent in other studies (Lottenberg *et al.*, 1992; Rustan *et al.*, 1993). However, the serum cholesterol was significantly lower in FO diet group than CP diet group and CF diet group tended to be low compared with CP diet group. This result suggests that n-3 long chain PUFA (*e.g.*, EPA, DHA) lowered the serum cholesterol strongly than n-6 PUFA and LNA. Similar results were observed in other studies (Lee *et al.*, 1989; Berr *et al.*, 1993). And the level of serum TG was tended to be low in the FO diet group. Therefore, the hypolipidemic effect of fish oil seems to result from the suppression of fatty acid synthesis and stimulation of peroxisomal β-oxidation, thus reducing availability of fatty acid substrate in serum lipid synthesis.

In conclusion, fish oil rich in n-3 EPA and DHA had smaller area of GST-P positive foci than n-6 linoleic acid and n-3 LNA. Different PUFAs seem to have different anticarcinogenic properties. Suppression of the hepatic lipogenesis and increase of peroxisomal β-oxidation may be considered to be a part of mechanism for preventive effect against chemical carcinogenesis and hypolipidemic effect of fish oil.

Table 5. Effect of dietary PUFA on lipid contents of liver and plasma in rat hepatocarcinogenesis

Group	Number of rats	Plasma cholesterol (mg/dl plasma)	Plasma triglyceride (mg/dl plasma)	Total lipid in liver (mg/g liver)	Cholesterol in liver (mg/g liver)	Triglyceride in liver (mg/g liver)
FO	5	52.70 ± 9.42^{b}	42.08 ± 11.59^{a}	81.14 ± 3.41^{a}	3.49 ± 0.13^{a}	6.79 ± 0.60^{a}
CF	5	66.26 ± 7.38^{ab}	63.92 ± 18.75^{a}	50.25 ± 7.31^{a}	2.23 ± 0.31^{b}	6.28 ± 0.39^{a}
CP	6	83.85 ± 3.54^{a}	76.75 ± 8.23^{a}	80.35 ± 12.26^{a}	3.66 ± 0.38^{a}	6.60 ± 0.41^{a}

FO, diet containing a fat mixture composed of fish oil and corn oil (8.2 : 1.8)+carcinogen treatment (DEN+partial hepatectomy); CF, diet containing a fat mixture composed of corn oil, beef tallow and fish oil (5 : 3.5 : 1.5)+carcinogen treatment (DEN+ partial hepatectomy); CP, diet containing a fat mixture composed of corn oil, beef tallow and perilla oil (5 : 4 : 1)+carcinogen treatment (DEN+partial hepatectomy). Values are mean±SE. Means with the same subscript are significantly different at $p<0.05$ by Duncan's multiple range test.

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