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Dietary polyunsaturated fatty acids suppress acute hepatitis, alter gene expression and prolong survival of female Long-Evans Cinnamon rats, a model of Wilson disease

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Abstract

In the Long-Evans Cinnamon rat, copper accumulates in the liver because of a mutation in the copper-transporting ATPase gene, and peroxidative stresses are supposed to be augmented. We examined the effects of dietary fatty acids on hepatitis, hepatic gene expression, and survival. Rats were fed a conventional, low-fat diet (CE2), a CE2 diet supplemented with 10 wt% of lard (Lar), high-linoleic soybean oil (Soy), or a mixture of docosahexaenoic acid (DHA)–rich fish oil and soybean oil (DHA/Soy). Among female rats, the mean survival times of the DHA/Soy and the Soy groups were longer by 17∼20% than in the Lar and the CE2 groups. Among male rats, the survival times were much longer than in the females, but no significant difference in survival was observed among the dietary groups. Serum ceruloplasmin levels in female and male rats of all of the dietary groups were similar. Serum transaminase levels of the DHA/Soy group tended to be lower than in the CE2 group. Histological examinations revealed a marked degeneration in hepatic tissue integrity in the Lar and CE2 groups but not in the DHA/Soy group. Hepatic levels of metal-related genes, transferrin and ceruloplasmin, as well as those related to bile acid synthesis were up-regulated, and an inflammation-related gene (cyclooxygenase [COX]–2) was down-regulated in the DHA/Soy group. Some proliferation-related genes were also affected by the dietary fatty acids. These results indicate that polyunsaturated fatty acids suppress the development of acute hepatitis and prolong survival in females, regardless of whether they are of the n-6 or n-3 type, which are associated with altered gene expressions.

Keywords: LEC rat; Longevity; Docosahexaenoic acid (DHA); Hepatitis; Microarray; Wilson disease; Peroxidative injury

1. Introduction

Long-Evans Cinnamon (LEC) rats are mutants that spontaneously develop acute hepatitis, and about one-half of the rats die of fulminant hepatitis. Those that survive acute hepatitis develop chronic hepatitis leading to hepatocellular carcinogenesis [1]. After 1.5 years of age, almost 100% of the surviving rats of both sexes manifest liver cancer [2]. Excessive accumulation of copper in the liver has been shown to be due to a mutation of a rat gene homologous to the human Wilson disease gene, ATP7B, which encodes a copper-transporting P-type ATPase [3]. The mutation of the ATP7B gene results in abnormal copper accumulation [4], reduced biliary copper excretion [5], decreased serum ceruloplasmin and copper levels [6], and increased hepatic iron accumulation [7,8]. The accumulated copper and iron are assumed to accelerate the formation of harmful hydroxyl radicals [9] that leads to the development of chronic hepatitis and then to carcinogenesis [2]. In humans, an increased incidence of hepatic carcinogenesis has been reported to be associated with chronic hepatitis and cirrhosis caused by hepatitis viruses [10]. Thus, the regulation of free-radical injury and chronic inflammation is of major importance in this model and in Wilson disease.

Polyunsaturated fatty acids are easily auto-oxidized in the air and may accelerate free-radical injury and subsequent inflammation in general. Alternatively, n-3 polyunsaturated fatty acids may trap free radicals and suppress

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ischemia and inflammation caused by overproduction of n-6 eicosanoids, arachidonic acid metabolites [11–14]. Thus, the dietary effects of polyunsaturated fatty acids of n-6 and n-3 types could not be easily predicted in LEC rats in which excessive amounts of copper and iron are accumulated in the liver. In fact, we have observed no significant difference in effects of hepatitis between linoleic acid (18:2n-6)–rich safflower oil and α-linolenic acid (18:3n-3)–rich perilla oil; both suppressed the development of acute hepatitis compared with a conventional, low-fat diet (CE2) [15]. To clarify in more detail the effect of type of dietary fatty acids on hepatitis-related markers in LEC rats, we examined the effects of a long-term feeding with fats and oils on hepatitis, hepatic histology, hepatic gene expression, and survival. Docosahexaenoic acid (DHA, 22:6n-3)-rich fish oil, linoleic acid-rich soybean oil and lard enriched with saturated and monounsaturated fatty acids were compared in relation to peroxidative indices (PIs) of the major fatty acids, using a conventional, low-fat diet as a control.

2. Methods and materials

2.1. Animals and diets

LEC rats and control Wistar-King Aptekman (WKA) rats were raised under specific pathogen-free (SPF) conditions. In each dietary group, 12 female and 12 male rats were used for the measurement of survival time, and 12 female rats were used for the measurements of biochemical parameters and histological examinations. We started feeding with test diets at 28 days of age. The basal diet was a conventional one (CE2, Central Laboratory for Experimental Animal [CLEA] Japan, Inc., Tokyo) containing 4.4% (w/w) lipids. The test fat or oil was prepared as described previously [16]. Lard (commercially available for human consumption, designated Lar), soybean oil (high-LA type, commercially available for human consumption, designated Soy), and a 1:9 mixture of fish oil (approximately 45% DHA, from Japan Oil Chemicals Inc., Tokyo) and soybean oil (designated DHA/Soy) were used, and the final lipid content was calculated to be 14.0 wt% (31.4 energy %). The fatty acid compositions of the experimental diets are shown in Table 1. The peroxidizability index (PI) was calculated as: PI = (% monoenoate × 0.025) + (% dienoate × 1) + (% trienoate × 2) + (% tetraenoate × 4) + (% pentaenoate × 6) + (% hexaenoate × 8) [17]. The DHA/Soy diet was replaced every day and other diets were replaced every 2 days to keep the peroxide values of the ingested diets to <10 mEq/kg.

Table 1
Fatty acid composition of experimental diet*

Fatty acid	CE2	Lar	Soy	DHA/Soy
	% of total fatty acid			
14:0	0.9	1.6	0.4	0.6
16:0	16.8	25.3	13.5	14.0
18:0	2.3	11.3	3.7	3.8
20:0	0.3	0.2	0.3	0.3
22:0	0.2	0.0	0.2	0.2
Total S:A	20.5	38.4	18.1	18.9
16:1†	1.3	2.1	0.5	0.7
18:1n-9†	23.2	37.2	25.2	22.4
18:1n-7†	0.9	0.1	0.0	0.1
Total 18:1†	24.1	37.3	25.2	22.5
20:1†	1.3	0.8	0.5	0.5
22:1†	0.5	0.0	0.1	0.1
Total MUFA	27.2	40.2	26.2	23.8
18:2n-6†	44.8	18.9	49.0	46.9
20:4n-6†	0.1	0.1	0.0	0.2
Total n-6	44.9	19.0	49.0	47.2
18:3n-3†	3.5	1.3	5.8	5.5
20:5n-3†	2.5	0.7	0.7	1.4
22:6n-3†	1.8	0.5	0.2	3.2
Total n-3	7.7	2.4	6.8	10.1
Total PUFA	52.4	21.4	55.8	57.3
n-6/n-3 ratio	5.8	7.9	7.3	4.7
PI‡	81.5	30.8	67.5	93.5
Total fatty acid (g/Kg Diet)	44.0	140.0	140.0	140.0

* The fatty acid composition of the diet (w(g)/100(g)) was analyzed by gas-liquid chromatography.

† The position of the first double bonds numbered from the methyl terminus is designated as n-9, n-7, n-6, or n-3.

‡ PI = peroxidizability index (see Reference [17]).

2.2. Biochemical analyses

The serum ceruloplasmin content was estimated essentially according to Sunderman and Nomoto [18]. Briefly, 2 mL of 0.1 mol/L acetate buffer (pH 5.2) was mixed with 0.1 mL of serum. The mixture was then incubated in a water bath at 37°C for 10 minutes, and 1 mL of 27.6 mmol/L PPD (*p*-phenylenediamine dihydrochloride) in 0.1 mmol/L acetate buffer (pH 5.2) was added to the test tube. After incubation for 5 minutes (blank) or 30 minutes (sample) at 37°C, 50 μL of sodium azide solution (0.1 mg/mL) was added to the tube. The absorbance at 530 nm was measured and the oxidase activity was obtained by subtracting the absorbance at 5 minutes (blank sample) or 30 minutes (test sample). Transaminase activities (aspartate aminotransferase [AST] and aspartate aminotransferase [ALT]) in the serum were determined using transaminase assay kits (Wako, Pure Chemicals Co., Osaka, Japan). Fatty acids were analyzed by gas–liquid chromatography as described previously [16].

2.3. Histological evaluation

Livers were excised, fixed with 10% neutral-buffered formalin, paraffin-embedded, sectioned, and then stained with hematoxylin and eosin. All histological studies were performed in a blind manner.

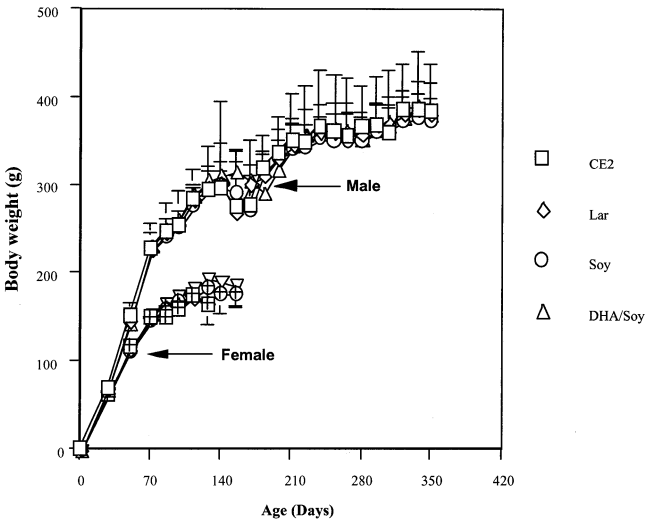

Fig. 1. Body weight of rats fed the CE2, Lar, Soy, or DHA/Soy diet. Test diets were fed from 28 days of age. The numbers of female and male rats were 12 in each group. Values with different letters indicate significant difference according to analysis of variance followed by Bonferroni/Dunn test at $P < 0.05$. Bars represent SD values.

2.4. Reverse transcription–polymerase chain reaction analysis

Cyclooxygenase-1 and -2 (COX-1 and COX-2) mRNAs were measured by Reverse transcripatse–polymerase chain reaction (RT-PCR) using a Superscript RT-PCR One-Step kit from Invitrogen Corp. (Carlsbad, CA). The cDNA was synthesized by reverse transcription reaction from 0.5 μg of total RNA isolated from the rat liver using the TRIZOL reagent (Gibco). The extracts were amplified by polymerase chain reaction (PCR) using 2 μL of RT reaction products in PCR buffer. The PCR cycling conditions were as follows: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, elongation at 72°C for 1 minute, and extension at 72°C for 5 minutes. Samples were collected after 30 cycles. The primers used for COX-1 were sense 5′-GCCGAGGATGTCATCAAGGAGTCCC-3′ and antisense 5′-ACGAAATCTCAAGATGGGCCCCGAC-3′. The primers used for COX-2 were sense 5′-CTTGTACGTCAGATTGCTGCCCGTAG-3′ and antisense 5′-CGAACCGAACACTGAAACCGTCGA-3′. The primers used for β-actin were sense 5′-CAGAGCAAGAGAGGCATCCT-3′ and antisense 5′-AGGATCTTCATGAGGTAGTC-3′. After amplification, each PCR product and a ladder of known molecular weights were electrophoresed in a 1% agarose gel containing 1 mg/mL ethidium bromide.

2.5. Microarray analysis

Total mixed RNAs of livers from three rats in each group were prepared and purified with the TRIZOL reagent (Gibco). The microarray analysis was performed using At-

Table 2
Fatty acid composition of total liver lipid in female LEC rats

Fatty acid	CE2	Lar	Soy	DHA/Soy
14:0	0.2 ± 0.1	0.2 ± 0	0.1 ± 0.1	0.1 ± 0
16:0	16.3 ± 1.2*	14.6 ± 0.5†	12.5 ± 0.4‡	13.8 ± 0.9†‡
18:0	13.5 ± 2.8†	14.4 ± 3.2†	21.8 ± 1.8*	21.0 ± 2.3*
20:0	0.2 ± 0.1*†	0.3 ± 0.1*	0.1 ± 0.1*†	0.1 ± 0†
22:0	0.1 ± 0.1	0.0 ± 0	0.0 ± 0	0.0 ± 0
24:0	0.4 ± 0.2†	0.4 ± 0.1†	0.8 ± 0.1*	0.7 ± 0.1*
Total SFA	30.8 ± 2.0†	30.0 ± 2.9†	35.4 ± 2.1*	35.8 ± 2.8*
16:1	0.8 ± 0.3*	0.4 ± 0.1†	0.2 ± 0.1†	0.3 ± 0.2†
18:1 n-9	11.0 ± 2.7†	17.0 ± 3.8*	6.1 ± 1.2‡	5.9 ± 1.2‡
18:1n-7	1.6 ± 0.8	0.5 ± 0.8	1.2 ± 0.6	1.0 ± 0.5
Total 18:1	12.6 ± 2.1†	17.5 ± 3.7*	7.3 ± 0.8‡	6.9 ± 0.9‡
20:1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0	0.2 ± 0
Total MUFA	14.1 ± 2.3†	18.5 ± 3.7*	8.5 ± 0.9‡	8.2 ± 1.3‡
18:2 n-6	22.7 ± 1.9*	14.5 ± 0.7†	20.3 ± 2*	21.3 ± 2*
20:4 n-6	10.6 ± 3.5†	11.4 ± 4.1†	19.9 ± 1.8*	16.6 ± 2*
22:4 n-6	0.2 ± 0.1*†	0.3 ± 0.1*	0.2 ± 0*†	0.1 ± 0†
22:5 n-6	0.1 ± 0.1†	0.2 ± 0.1*†	0.3 ± 0.1*	0.3 ± 0.1*
Total n-6	33.5 ± 2.0†	26.4 ± 3.8‡	40.7 ± 0.9*	38.2 ± 0.7*
18:3 n-3	0.6 ± 0.1†	0.3 ± 0.1‡	0.9 ± 0.1*	0.9 ± 0.2*
20:5 n-3	0.7 ± 0.2	0.4 ± 0.2	0.4 ± 0.1	0.7 ± 0.1
22:5 n-3	1.2 ± 0.2*	0.9 ± 0.2†	0.6 ± 0‡	0.1 ± 0.1§
22:6 n-3	6.4 ± 0.7†	6.0 ± 1†	6.3 ± 0.5†	9.2 ± 0.8*
Total n-3	9.0 ± 0.7†	7.7 ± 0.9†	8.2 ± 0.6†	10.9 ± 0.9*
n-6/n-3 ratio	3.7 ± 0.2†	3.4 ± 0.3†	5.0 ± 0.4*	3.5 ± 0.3†
PI	131 ± 17.1†	119 ± 23†	161 ± 5.2*	170 ± 6.1*
Total FA (mg/g)	43.9 ± 5.7*	41.5 ± 5.7*†	33.1 ± 6.8†‡	31.6 ± 3.1‡

Fatty acid composition of total hepatic lipid (% of total fatty acids) is shown as mean ± SD ($n = 6$). Rats at 105 days of age were used.
*,†,‡,§ Values with different superscripts are significantly different from each other at $P < 0.05$.

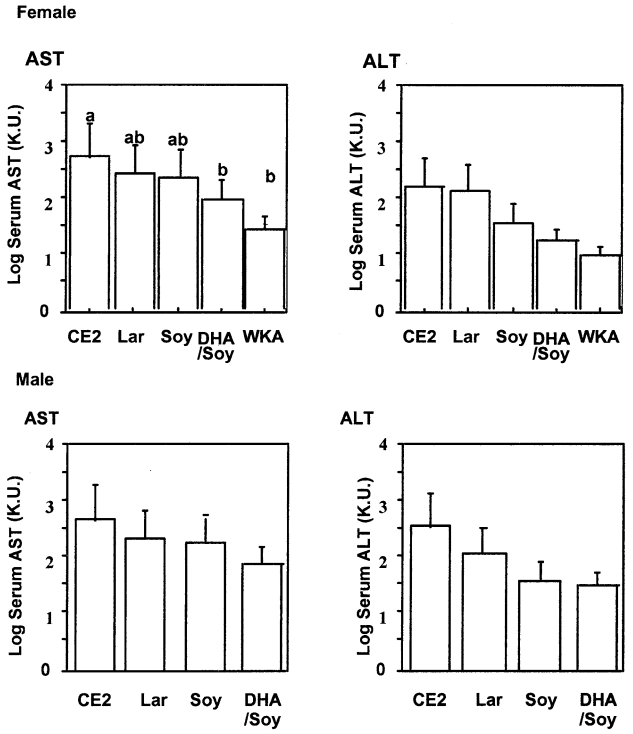

Fig. 2. Effect of dietary oils on serum AST, and ALT in LEC rats. Values are means ± SD ($n = 6$ for the CE-2, Lar, Soy and DHA/Soy groups of female rats; $n = 3$ for WKA and male groups). Rats at 105 days of age were analyzed. Values with different superscripts are significantly different from each other as determined by analysis of variance at $P < 0.05$.

las™ Glass Rat 1.0 Microarray (microarray 1) (Clontech Laboratories, Inc., Palo Alto, CA) and Rat Liver Array Ver. 1.0 (microarray 2) (Nippon Laser & Electronics Laboratory, Nagoya, Japan) according to the manufacturer's instructions. Individual mRNA levels were scanned as intensities of Cy3 (532 nm) and Cy5 (635 nm) fluorescence (Amersham Pharmacia Biotech Ltd., Little Chalfont, UK) using a GenePix 4000 (Axon Instruments Inc., Foster City, CA) or GTMAS SCAN II (Nippon Laser & Electronics Laboratory), and then scored and analyzed by GenePix pro 3.0 (Axon Instruments Inc., Foster City, CA) or Array–Pro Analyzer (Media Cybernetics, Silver Spring, MD). Tissue samples from day 105 were analyzed.

2.6. Western blotting

Immunoblotting was performed essentially as described previously [19].

2.7. Statistical analyses

Data were presented as means ± SD. Statistical analysis of the survival rates was performed by using the log rank and Wilcoxon signed rank method (a nonparametric method) using the computer program JMP 3.0, Statistics Made Visual (SAS Institute, Inc., Cary, NC). Other data were analyzed using Bonferroni's multiple comparison (Stat View version J-4.11; Abacus Concepts, Inc., Berkeley, CA).

3. Results

3.1. Growth and fatty acid composition of hepatic total lipids

The body weight of male rats was significantly higher than that of female rats from day 42 (Fig. 1). However, there was no significant difference among the dietary groups of the male or female rats. Weight loss was observed after ~140 days of age in all dietary groups of male rats, the time when rats began to die.

The fatty acid composition of total lipids in the liver roughly reflected that of the diet (Table 2). The total fatty acid content and the proportion of monounsaturated fatty acids tended to be greater, whereas the proportion of saturated fatty acids was less in the CE2 and Lar groups than those in the Soy and DHA/Soy groups. The proportions of arachidonic acid (n-6) and total n-6 fatty acids were significantly less in the CE2 and Lar groups, whereas the proportion of DHA (22:6n-3) was greater in the DHA/Soy group than in the other groups. The differences in the fatty acid composition of the dietary groups were less in male rats (date not shown). The peroxidizability indices (PIs), which are measures of susceptibility to auto-oxidation in the air atmosphere, were significantly higher in the DHA/Soy and Soy groups than in the other two groups in both the female and male rats.

3.2. Biochemical parameters in serum

Serum AST activity decreased in the order of the CE2, Lar, Soy, and DHA/Soy groups of female rats on day 105 (Fig. 2). ALT activities in the CE2 and Lar groups were higher than in the Soy and DHA/Soy groups. Male rats exhibited similar differences in transaminase activities among the dietary groups, but the difference among the dietary groups was not statistically significant (Fig. 2).

Reduction in the level of serum ceruloplasmin activity, an α-globulin that binds to six copper atoms, is a diagnostic parameter of Wilson disease. The levels of serum ceruloplasmin activity in the CE2, Lar, Soy, and DHA/Soy groups of both male and female rats were significantly lower than that in the control WKA rats fed CE2 diet, but these differences among the dietary groups of the LEC rat were relatively small (Table 3). Similarly, the levels of serum copper in the LEC rat were significantly lower than in the WKA rat, but no significant difference was observed among the dietary groups of the LEC rat. Serum iron and calcium levels in female rats were not different among the dietary groups (data not shown). These results taken together indicate that serum ceruloplasmin and metal levels in the female LEC rat are influenced relatively little by the amounts and compo-

Table 3
Effect dietary oils on biochemical parameters and hepatic histology

Strain	LEC Rat				WKA Rat
Female/Diet group*	CE2	Lar	Soy	DHA/Soy	CE2
Serum ceruloplasmin (μg/dL)	0.059	0.047	0.051	0.034	1.130
Serum copper (μg/dL)	38	40	36	42	160
Rats with jaundice	24/24	22/24	4/24	0/24	ND
Hepatocellular change	+++	+++	++	+	ND
Cholestasis	+	+	±	−	ND
Atypical regeneration	+++	+++	±	±	ND
Fatty change	++	+	±	±	ND
Male/Diet Group	CE2	Lar	Soy	DHA/Soy	CE2
Serum ceruloplasmin (μg/dL)	0.088	0.051	0.029	0.018	1.250
Serum copper (μg/dL)	52	45	50	48	169
Rats with jaundice	10/24	8/24	7/24	724	ND
Hepatocellular change	++	++	+	−	ND
Cholestasis	+	+	±	−	ND
Atypical regeneration	+++	+++	±	±	ND
Fatty change	++	+	+	+	ND

* Serum ceruloplasmin, hepatocellular change, cholestasis, atypical regeneration, and fatty change in each dietary group at 105 days of age were measured ($n = 6$ for female LEC rat; $n = 3$ for male LEC rats and WKA rats) and the severity was shown in 5 ranks from no change (−) to the most severe change (+++). Three rats were assigned for measurement of serum copper ($n = 3$ for LEC rats and WKA rats). Twenty-four rats were used for measurement of rats with jaundice in each dietary group. Values are means ± SD. ND = not determined.

sitions of dietary fatty acids, but are significantly lower than in the control WKA rat.

3.3. Histological examination of liver and evaluation of jaundice

Acute hepatitis in the LEC rat usually begins at approximately 95–126 days of age, and about one-half of rats with jaundice subsequently die of hepatic failure [4]. We evaluated jaundice based on yellowing of the ear and tail. At 105 days of age, most of the female rats were jaundiced in the CE2 and Lar groups, but the incidence of jaundice in the Soy and DHA/Soy groups was very low (Table 3). In male LEC rats on day 105, the incidence of jaundice was less than 50% in all dietary groups. Histologically, destruction of liver tissue integrity was observed in the CE2 and Lar groups, both female and male. Hepatic cells, together with infiltrating leukocytes and hemorrhage, were more frequent, and in the Lar group the hepatic cells exhibited a marked increase in lipid droplets as compared with the DHA/Soy group. The histological changes in the Soy group were much less than in the CE2 and Lar groups. These observations were presented in a semiquantitative manner in Table 3. These results indicate that the DHA/Soy and Soy diets suppress the onset of hepatitis, and suggest that the suppression is not simply due to competitive inhibition of inflammatory n-6 eicosanoid synthesis by DHA (n-3).

3.4. Suppression of lethal hepatitis by polyunsaturated fatty acids

The mean survival time in the female decreased in the order of the DHA/Soy (>202 days), Soy (165 days), Lar (128 days), and CE2 (125 days) groups as shown in Fig. 3. Statistically, at 180 days of age, the survival time of the females in the DHA/Soy group was longer by 20% ($P < 0.008$) than in the CE2 group and by 17% ($P < 0.01$) than in the Lar group. In the male rats at day 500, the survival times decreased in the order of the DHA/Soy (>374 days), Soy (>359 days), CE2 (343 days), and Lar group (319 days), but these differences were not statistically significant.

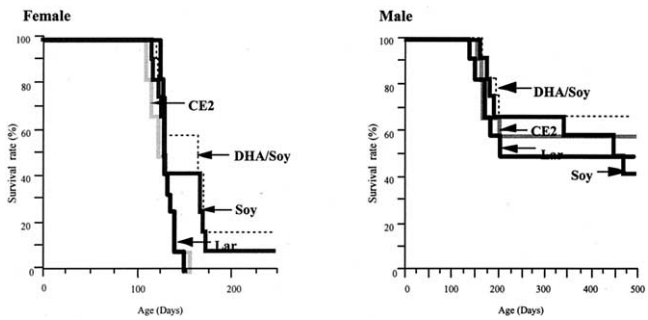

Fig. 3. Effect of dietary oils on survival rate of rats. Rats were fed experimental diets from 28 days of age. The number of rats was 12 in each group.

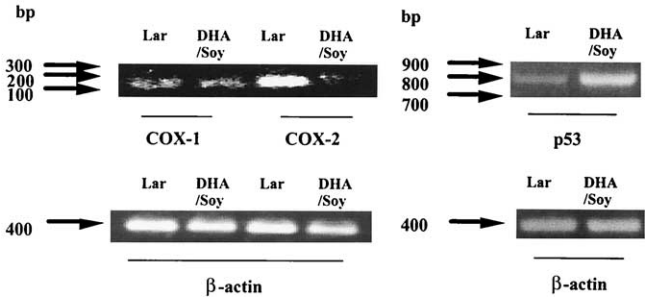

Fig. 4. Effect of dietary oils on hepatic COX-1, COX-2, and p53 mRNA expression in female rats. For the Lar and DHA/Soy groups, livers from rats at 105 days of age were used for the determination of COX-1, COX-2, and p53 mRNA expression levels.

3.5. Profile of hepatic mRNA expression

To investigate the possible correlations between dietary effect on hepatitis and gene expression [20,21] in the LEC rat, we used microarray analysis, and only the DHA/Soy and the Lar groups were compared. Of 1090 mRNAs, >65 decreased (<0.7-fold) and >398 increased (>1.2-fold) in the DHA/Soy group compared with the Lar group. As has been well characterized in relatively short-term feeding experiments, long-term feeding of DHA/Soy diet (105 days) elevated mRNA expression levels of enzymes involved in fatty acid oxidation and energy metabolism such as pancreatic lipase–related protein 2 (1.4-fold), hepatic triacylglycerol lipase (1.9-fold), pancreatic triacylglycerol lipase (2.8-fold), liver fatty acid-binding protein (2.1-fold), liver fatty acid-binding protein 5 (1.8-fold), fatty acid-binding protein 9 (2.4-fold), long-chain acyl-CoA synthetase 2 (1.4-fold), long-chain–specific acyl-CoA dehydrogenase (2.1-fold), short-chain–specific acyl-CoA dehydrogenase (1.3-fold), and acyl-CoA oxidase (2.5-fold).

Among the metal-related genes, the level of transferrin receptor mRNA for iron transport was elevated (1.9-fold), and the ceruloplasmin mRNA expression level was slightly elevated (1.8-fold) in the DHA/Soy group compared with those in the Lar group. Copper absorbed from intestine is transported to tissues as a protein-bound form and is excreted from the liver mainly through the bile into the duodenum. The expression levels of the mRNA of the key regulatory enzymes in bile acid synthesis, namely, cytochrome p450 VII (CYP7) (2.1-fold), CYP27 (2.0-fold), and taurine transporter (2.1-fold), were also elevated. The expression levels of other cytochrome p450 species (CYP2A3, CYP2C7, CYP4A3, CYP4A8, cytochrome p450 2J3, CYP3A9, CYP2E1) were also elevated (1.6-fold).

Among proliferation- and apoptosis-related genes, the genes presumed to suppress cellular proliferation were up-regulated; prohibitin (1.7-fold), cellular tumor antigen p53 (1.6-fold), and BCL-2-like protein 1 (BCL-X) (4.2-fold) in the DHA/Soy group compared with those in the Lar group. The difference in p53 mRNA expression of the two dietary groups was assessed by an RT-PCR method (Fig. 4).

Inflammation-related genes were further evaluated by RT-PCR. The COX-2 mRNA expression level in the DHA/Soy group was significantly lower than that in the Lar group (Fig. 4). The COX-1 mRNA expression levels were not different between the two groups. The protein expression levels of COX-2 were evaluated by immunoblotting. Similar differences in the protein expression level between the two dietary groups were detected (data not shown).

4. Discussion

Compared with saturated and monounsaturated fatty acids that are present in animal fats, polyunsaturated fatty acids such as eicosapentaenoic acid and DHA in seafood are known to suppress gene expressions of lipogenic and cholesterol synthetic enzymes through sterol-responsive element-binding proteins (SREBPs) [21,22], and to stimulate the expression of genes involved in β-oxidation and thermogenesis (uncoupling proteins) through peroxisome-proliferator activated receptors [23–26]. These differences were qualitatively reproduced in the DHA/Soy and the Lar diets in the present long-term feeding experiments, although the observed differences appear to be much less than those seen in cultured cells [27,28]. Arachidonic acid (n-6) is a minor component in our foods, but is produced from dietary linoleic acid to exert effects on gene expressions similarly to eicosapentaenoic acid (n-3) and DHA (n-3) [29,30]. Consistently, both the DHA/Soy and Soy diets increased DHA and arachidonic acid in hepatic lipids, suppressed hepatic injury, and prolonged survival compared with the CE2 and the Lar diets. It is also possible that polyunsaturated fatty acids (PUFAs) served as scavengers rather than proliferators of free radicals produced in the liver of LEC rats.

We interpret that the mutation of the ATP7B gene (Cu-binding ATPase for efflux of Cu) reduced biliary copper excretion, resulting in abnormal copper accumulation in the liver (Fig. 5). Compared with the Lar diet, the DHA/Soy diet increased the expression of hepatic ceruloplasmin (1.6-fold), although serum copper as well as serum ceruloplasmin levels were essentially unchanged. Some genes involved in biosynthesis of bile acid from cholesterol were up-regulated in the DHA/Soy group compared with those in the Lar group, which is consistent with the observations that dietary fish oil stimulates bile acid secretion [30]. The inability of LEC rats to excrete copper into the bile has been suggested as a possible cause of enhanced cholestasis, jaundice, hepatitis, and hepatic carcinogenesis [5,31]. Reduced expression levels of hepatobiliary transport systems for bile salts and other organic anions have also been shown to contribute to inflammation-inducing cholestasis in humans [32,33].

N-3 PUFAs inhibit inflammation and some types of cancer through competitive inhibition of n-6 eicosanoid metabolism [11]. Hepatic inflammation measured as transaminase activities was severer in the Lar group than in

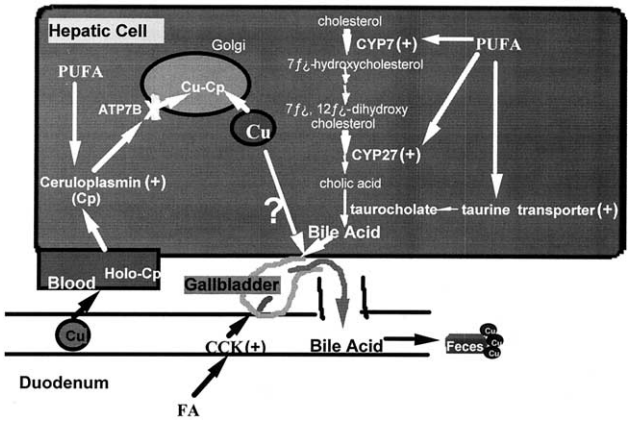

Fig. 5. Possible correlation of dietary fatty acids with hepatic copper metabolism. The LEC rat has a defect in the ATP7B gene (Cu-binding ATPase for efflux of Cu). Dietary polyunsaturated fatty acids (PUFAs) up-regulated the gene expressions of hepatic ceruloplasmin and bile acid–related proteins (CYP7, CYP27, and taurine transporter), although serum copper and ceruloplasmin levels were essentially unchanged. Fatty acids (FA) are known to stimulate the release of cholecystokinin (CCK) hormone from the intestinal mucosa to contract gallbladder and secrete bile acids into the duodenum, and also that fish oil stimulate bile acid secretion [30]. Thus, it is speculated that PUFAs may increase bile acid synthesis and excretion of copper through biliary pathway to feces.

the DHA/Soy and Soy groups. The COX-2 expression level was elevated in cases of inflammation [34–36], and the COX-2 mRNA expression level in the Lar group was higher than that in the DHA/Soy group. The proposed COX-independent pathway [37] and/or the pathway through peroxisome-proliferator activated receptors may also be involved in hepatic inflammation, contributing to the differences observed between the Lar and DHA/Soy groups.

Contrary to other rat strains such as the stroke-prone SHR rat, the mean survival time was much longer in male than in female LEC rats (Fig. 3). A simple speculation is that there may be genes on Y-chromosome that compensate for the defect of the ABC transporter (ATPB7), although we have no evidence that this is the primary defect in this animal model. As to sex-related hormones, aromatase, a key enzyme for estrogen synthesis, was down-regulated in the DHA/Soy group (6-fold) compared with the Lar group, which may have contributed to suppress the hepatic injury. In fact, suppression of estrogen synthesis in female rats is suggested to reduce estrogen nuclear receptor activity and to decrease breast cancer incidence [38]. Suppression by DHA/Soy diet of proliferation-related genes (prohibitin) is also likely to have served to suppress the progress of hepatic injury leading to carcinogenesis. However, up-regulation of genes related to apoptosis, cellular tumor antigen p53, BCL-2-associated X protein membrane isoform alpha (Bax-α), BCL-2, and BCL-X in the DHA/Soy group could not be fully understood from our current knowledge.

In summary, the observed differences in the progression of hepatitis and survival time among the dietary groups of the LEC rat could not be accounted for simply by competition between n-6 and n-3 fatty acids to produce inflammatory lipid mediators. Instead, differential effects on gene expressions by these dietary fatty acids as well as possible scavenger effects of PUFAs appeared to play more important roles. Obviously, further studies are necessary to clarify the mechanisms by which both n-6 and n-3 PUFAs extend the survival of female LEC rats.

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