The Effect of Chemical Hepatocarcinogenesis on Liver Phospholipid Composition in Rats Fed N-6 and N-3 Fatty Acid-Supplemented Diets (43370)

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Abstract. **The effect of dietary fats on essential fatty acid metabolism in rats subjected to chemically induced hepatocarcinogenesis was studied. Sixty male rats were fed a diet supplemented with one of the following three oil compositions: 10% hydrogenated coconut oil (HCO); 5% hydrogenated coconut oil and 5% γ-linolenic acid (18:3n-6)-rich evening primrose oil (EPO); or 5% hydrogenated coconut oil and 5% marine oil (FO). Half of the animals in each dietary regimen were subjected to hepatocarcinogenesis induction using diethylnitrosamine and 2-acetylaminofluorene (2-AAF) followed by partial hepatectomy, whereas the other half underwent hepatectomy without receiving diethylnitrosamine and 2-acetylaminofluorene. Liver phospholipid composition was analyzed. In comparison to the HCO group, the EPO group showed raised levels of arachidonic acid (20:4n-6) and suppressed n-3 fatty acids. The FO group, on the other hand, showed suppressed levels of n-6 and increased n-3 fatty acids. Hepatocarcinogenesis suppressed the level of 20:4n-6 and this effect was greater in the FO rats. The levels of dihomo-γ-linolenic acid (20:3n-6) were increased by the hepatocarcinogenic treatment, and this effect was further accentuated in the EPO rats. These results suggest that hepatocarcinogenesis may suppress the activity of Δ-5-desaturase, which may be one of the reasons why tumor cell membranes have low levels of long chain fatty acids, especially 20:4n-6 cells, and have an impaired capacity to undergo lipid peroxidation.** [P.S.E.B.M. 1992, Vol 199]

The model for the chemical induction of hepatocellular carcinoma in rats, as developed by Solt and Farber (1), consists of a combined regimen of the carcinogens diethylnitrosamine (DEN) and 2-acetylaminofluorene (2-AAF) followed by partial hepatectomy. Pretreatment with carcinogens predisposes the hepatic tissue to neoplastic change and the development of small hyperplastic foci or nodules. These premalignant nodules, which originate by dedifferentiation of adult liver cells, are believed to progress to hepatocarcinomas within 36 weeks of the carcinogenic treatment (2). Benedetti *et al.* (3) have shown that these foci have a reduced capacity for lipid peroxidation and suggested that loss of lipid peroxidation could also be an early event in hepatocarcinogenesis. This view is supported by the observation that the rate of lipid peroxidation is generally low in tumor cells (4). Polyunsaturated fatty acids, which are known to influence tumor cell growth and survival, are the main substrates for lipid peroxidation (4, 5). Both low polyunsaturated fatty acid levels and low lipid peroxidation could be due to abnormal essential fatty acid metabolism (6). In this study, we examine the effect of dietary supplementation with n-3 and n-6 polyunsaturated fatty acids on liver polyunsaturated fatty acid distribution in animals subjected to chemical hepatocarcinogenesis.

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Materials and Methods

Reagents and Equipment. DEN and 2-AAF were purchased from Sigma Chemical Co. (St. Louis, MO).

Chloroform, methanol, butylated hydroxytoluene, hexane, diethyl ether, acetic acid, and silica gel 60 thin layer chromatography plates were purchased from BDH Chemicals Canada (Dartmouth, NS, Canada). Boron fluoride was obtained from Supelco Canada Inc. (Oakville, Ontario, Canada). Teflon-capped vials were purchased from Fisher Scientific Ltd. (Dartmouth, NS, Canada). The Hewlett Packard 5880A gas liquid chromatography apparatus using Gas Chrom Q column was obtained from Hewlett Packard (Canada) Ltd. (Mississauga, Ontario, Canada).

Animals. Sixty male Fischer 344 rats weighing approximately 250 g, were obtained from the Charles River Breeding Laboratories Inc. (Montreal, Quebec, Canada). The animals were randomly divided into three groups (n = 20), and were fed a fat-free semisynthetic diet (Teklad Test Diets, Madison, WI) supplemented with 10% by weight of oil. The first group received 10% hydrogenated coconut oil (HCO); the second group received a mixture of 5% hydrogenated coconut oil and 5% evening primrose oil (EPO), a rich source of linoleic (18:2n-6) and γ-linolenic acids (18:3n-6); and the third group received a mixture of 5% hydrogenated coconut oil and 5% fish oil (FO), which contains high amounts of eicosapentaenoic (20:5n-3) and docosahexaenoic acids (22:6n-3). The detailed fatty acid compositions of the oil combinations are presented in Table I. Half of the animals in each of the dietary groups were subjected to the Solt and Farber (1) procedure for the induction of liver hyperplastic nodules and subsequent hepatomas. Briefly, the animals were maintained on their respective diets until sacrificed. On the day the dietary regimens were started, they also received an intraperitoneal injection of DEN at a dose of 200 mg/kg body wt. Two weeks after the DEN administration, 2-AAF at a concentration of 0.02% was supplied to the animals in drinking water until the animals were sacrificed. A 70% partial hepatectomy was performed 3 weeks after the DEN treatment. The animals were sacrificed (between 9 and 10 AM) 10 days after the partial hepatectomy. The controls underwent the partial hepatectomy but received no DEN and 2-AAF treatment.

Table I. Fatty Acid Composition (mg/100 mg) of the Oil Combinations[a] Supplemented to the Fat-Free Semisynthetic Diet[b]

Fatty acid	HCO	EPO	FO
10:0	6.0	3.0	2.1
12:0	44.6	22.3	18.7
14:0	17.2	8.6	13.0
16:0	9.1	7.6	13.7
18:0	12.6	7.2	8.9
18:1 (n-9)	3.0	5.5	9.0
18:2 (n-6)	0.3	37.0	0.9
18:3 (n-6)	—	4.6	—
18:3 (n-3)	—	0.4	0.5
20:4 (n-6)	—	—	1.0
20:5 (n-3)	—	—	10.1
22:5 (n-3)	—	—	1.3
22:6 (n-3)	—	—	7.0

[a] HCO: 10% hydrogenated coconut oil; EPO: 5% hydrogenated coconut oil and 5% evening primrose oil; FO: 5% hydrogenated coconut oil and 5% fish oil.
[b] Diet contains: 60.2% sucrose, 20% vitamin-free casein, 10% fat, 5% cellulose, 3.5% mineral mix (AIN-76), 1% vitamin mix (Teklad, 40060), and 0.3% DL-methionine.

Fatty Acid Analysis. The animals were sacrificed under light ether anesthesia and the livers were rapidly excised, rinsed in cold saline, and frozen until analysis within 2 weeks. Phospholipids and other lipid fractions were separated by thin layer chromatography and methylated as described by Morrison and Smith (7). Fatty acid analyses were performed by gas liquid chromatography as described previously (8, 9). The apparatus consisted of a Hewlett Packard 5880A set-up housing a 10% Silar 10C on Gas Chrom Q column and connected to an automatic integrator. Two-way analysis of variance was used to assess the significance of the differences among the groups.

Results and Discussion

The composition of major n-3 and n-6 fatty acids in liver phospholipid is shown in Table II. In all three dietary groups, hepatocarcinogenic treatment significantly increased the levels of dihomo-γ-linolenic acid (20:3n-6), whereas it decreased those of arachidonic acid (20:4n-6). Eicosapentaenoic acid (20:5n-3) was not significantly affected by the hepatocarcinogenic treatment. Similar changes were also seen in the triglyceride fraction (data not shown). In comparison with HCO feeding, EPO feeding increased the levels of 20:4n-6, whereas it suppressed the levels of n-3 fatty acids. Those receiving FO showed a decrease in 20:4n-6 levels and a significant increase in the n-3 fatty acid levels. Upon treatment with the carcinogenic regimen, the increase in the 20:3n-6 level in the EPO group and the reduction of 20:4n-6 in the FO group were significantly accentuated. It is well established that supplementation of 18:3n-6 in the EPO group bypasses the Δ-6-desaturase rate-limiting step in 18:2n-6 metabolism. γ-Linolenic acid (18:3n-6) is elongated to 20:3n-6 which serves as a substrate for Δ-5-desaturase. On the other hand, 20:5n-3 in fish oil is known to suppress Δ-5-desaturase activity (8, 10). When the ratio of product to substrate in liver phospholipid was analyzed for reflecting activity of the desaturase as shown in Figure 1, it was evident that the carcinogenic treatment produced changes consistent with suppression of Δ-5-desaturase activity, as shown by the decreased 20:4n-6:20:3n-6 ratio. The ratio of 20:4n-6:18:2n-6, which represents cumulative Δ-5- and Δ-6-desaturase activities, is less reduced by hepatocarcinogenic treatment than Δ-5-desaturase activity alone, suggesting that the latter is the more affected process.

Table II. N-6 and N-3 Fatty Acid Composition (mg/100 mg) of Liver Phospholipid Fraction in Rats Fed a Fat-Free Semisynthetic Diet Supplemented with Hydrogenated Coconut Oil or Combinations of Hydrogenated Coconut Oil and Evening Primrose or Fish Oils With or Without Hepatocarcinogenic Treatment[a]

Fatty acid	HCO		EPO		FO		Significance
	−HC[b]	+HC	−HC	+HC	−HC	+HC	
18:2 (n-6)	14.7 ± 1.0	15.3 ± 0.3	13.0 ± 1.2	14.6 ± 0.8	12.0 ± 1.1	12.2 ± 2.0	<0.05
18:3 (n-6)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	ND	ND	NS
20:3 (n-6)	0.9 ± 0.2	1.5 ± 0.3	0.9 ± 0.2	1.9 ± 0.2	0.8 ± 0.1	1.1 ± 0.1	<0.01
20:4 (n-6)	22.6 ± 0.6	19.0 ± 1.5	28.5 ± 1.7	25.9 ± 1.6	15.3 ± 1.7	10.3 ± 0.7	<0.001
22:4 (n-6)	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	ND	0.1 ± 0.0	NS
22:5 (n-6)	0.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.1	0.6 ± 0.2	NS
18:3 (n-3)	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	<0.05
20:5 (n-3)	0.9 ± 0.2	0.8 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	6.6 ± 1.2	6.9 ± 1.0	<0.001
22:5 (n-3)	1.7 ± 0.6	2.0 ± 0.2	1.2 ± 0.2	1.3 ± 0.1	2.6 ± 0.1	3.7 ± 0.5	<0.001
22:6 (n-3)	7.4 ± 0.6	9.4 ± 0.6	6.2 ± 0.3	6.8 ± 0.2	13.8 ± 1.1	14.4 ± 1.5	<0.001

[a] Results are expressed as mean ± SD of 10 observations. Comparisons were made between respective dietary groups with (+HC) or without (−HC) hapatocarcinogenic treatment by means of two-way analysis of variance. For details, see footnotes to Table I.
[b] HC: Hepatocarcinogenic treatment indicates treatment with diethylnitrosamine, 2-acetylaminofluorine, and partial hepatectomy. ND, none detectable; NS, not significant.

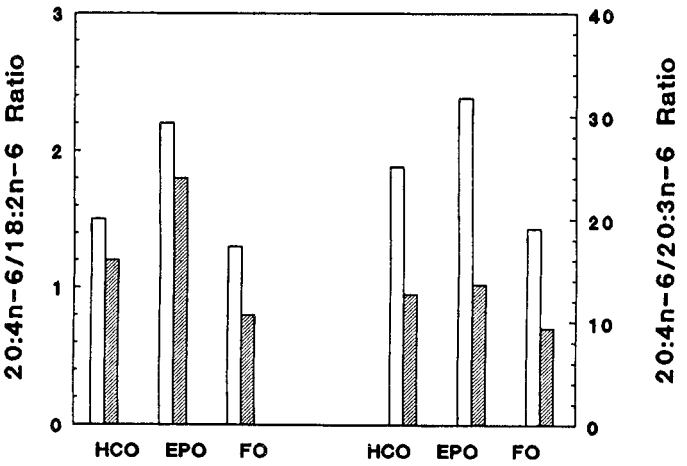

Figure 1. The ratio of product to substrate in the liver phospholipid fraction of rats fed a fat-free semisynthetic diet supplemented with 10% hydrogenated coconut oil (HCO); a combination of 5% hydrogenated coconut oil and 5% evening primrose (EPO); or a combination of 5% hydrogenated cocout oil and 5% fish oil (FO); with (shaded) or without (unshaded) heptocarcinogenic treatment. The 20:4n-6:18:2n-6 ratio reflects cumulative Δ-5- and Δ-6-desaturase activities, while the 20:4n-6:20:3n-6 ratio reflects mainly Δ-5-desaturase activity.

Cheeseman *et al.* (4) and others (11, 12) have shown that hepatic tumor cells contain significantly low amounts of polyunsaturated fatty acids, especially 20:4n-6. Furthermore, tissues undergoing rapid cell division, whether benign or malignant, also have a very low content of lipid peroxides (13). It is possible that the loss of lipid peroxidation seen in preneoplastic hepatic tissue (3) is the result of low polyunsaturated fatty acid levels in the tissue. The results of the present study suggest that hepatocarcinogens alter essential fatty acid metabolism by suppressing the Δ-5-desaturase activity.

In earlier studies, it has been shown that 20:3n-6, 20:4n-6, and 20:5n-3 acids selectively kill tumor cells by augmentation of production of free radicals in tumor cells as compared with normal cells (5, 14–16). Thus, the low rates of lipid peroxidation and free radical generation by tumor cells may, at least in part, be due to substrate deficiency, such as low content of 20:4n-6, which in turn is the result of a suppressive Δ-5-desaturase activity. These results suggest that Δ-5-desaturase may play a significant role in mutagenesis and carcinogenesis.

1. Solt D, Medline A, Farber E. Rapid emergence of carcinogen-induced hyperplastic lesions in a new model for the sequential analysis of liver carcinogenesis. Am J Pathol **88**:595–609, 1977.
2. Scherer E, Emmelot P. Foci of altered liver cells induced by a single dose of diethylnitrosamine and partial hepatectomy: Their contribution to hepatocarcinogenesis in the rat. Eur J Cancer **11**:145–154, 1975.
3. Benedetti A, Malvaldi G, Fulceri R, Comporti M. Loss of lipid peroxidation as an histochemical marker for preneoplastic hepatocellular foci in rats. Cancer Res **44**:5712–5717, 1984.
4. Cheeseman KH, Burton GW, Ingold KU, Slater TF. Lipid peroxidation and lipid antioxidants in normal and tumor cells. Toxicol Pathol **12**:235–239, 1984.
5. Begin ME, Das UN, Ells G, Horrobin DF. Selective killing of human cancer cells by polyunsaturated fatty acids. Prostaglandins Leukotrienes Med **19**:177–186, 1985.
6. Horrobin DF. Essential fatty acids, lipid peroxidation, and cancer. In: Horrobin DF, Ed. Omega-6 Essential Fatty Acids: Pathophysiology and Role in Clinical Medicine. New York: Alan R. Liss, p351, 1990.
7. Morrison WR, Smith LM. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. J Lipid Res **5**:600–608, 1964.
8. Nassar BA, Huang YS, Manku MS, Das UN, Morse N, Horrobin DF. The influence of dietary manipulation with n-3 and n-6 fatty acids on liver and plasma phospholipid fatty acids in rats. Lipids **21**:652–656, 1986.
9. Huang YS, Manku MS, Horrobin DF. The effects of dietary

cholesterol on blood and liver polyunsaturated fatty acids and on plasma cholesterol in rats fed various types of fatty acid diet. Lipids **19**:664–672, 1984.
10. Takahashi R, Nassar BA, Huang YS, Begin ME, Horrobin DF. Effects of different ratios of dietary n-6 and n-3 fatty acids on fatty acid composition, prostaglandin formation and platelet aggregation in the rat. Thromb Res **47**:135–146, 1987.
11. Morisaki N, Lindsey JA, Stitts JM, Zhang H, Cornwell DG. Fatty acid metabolism and cell proliferation. V: Evaluation of pathways for the generation of lipid peroxides. Lipids **19**:381–394, 1984.
12. Schroeder F, Gardiner JM. Membrane lipids and enzymes of cultured high- and low-metastatic B16 melanoma variants. Cancer Res **44**:3262–3269, 1984.
13. Wolfson N, Wilbur KM, Bernheim F. Lipid peroxide formation in regenerating rat liver. Exp Cell Res **10**:556–558, 1956.
14. Begin ME, Ells G, Das UN, Horrobin DF. Differential killing of human carcinoma cells supplemented with n-3 and n-6 polyunsaturated fatty acids. JNCI **77**:1053–1062, 1986.
15. Booyens J, Engelbrecht P, le Roux S, Louwrens CC, Van der Merwe CF, Katzeff IE. Some effects of the essential fatty acids linoleic acid and alpha linolenic acid and their metabolites gamma-linolenic acid, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid, and of prostaglandins A_1 and E_1 on the proliferation of human oestogenic sarcoma cells in culture. Prostaglandins Leukotrienes Med **15**:15–33, 1984.
16. Das UN, Huang YS, Begin ME, Ells G, Horrobin DF. Uptake and distribution of cis-unsaturated fatty acids and their effect on radical generation. J Free Radic Biol Med **3**:9–14, 1987.