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Different Effects of Carbon Tetrachloride on Carcinogen-induced Hepatocellular Carcinoma and Normal Liver of the Rat: Lowered Lipid Peroxidation and Accelerated Necrosis in Cancer

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To investigate molecular responses to lipid peroxidative stimuli in neoplastic cells, lipid peroxidation was induced in liver of rats bearing 3′-methyl-4-dimethylaminoazobenzene-induced hepatocellular carcinoma by injecting a high dose of carbon tetrachloride (CCl_4), a strong lipoperoxidative reagent. Normal rat livers with or without CCl_4 treatment served as controls. CCl_4 administration markedly provoked fatty metamorphosis, visualized by oil red O staining, in normal livers while minimal fatty changes were seen in hepatocellular carcinomas, where necrosis was often observed instead. After CCl_4 treatment, the thiobarbituric acid values (representing levels of lipid peroxides in the tissue) were increased two-fold in the untreated normal liver, but were unchanged in the cancer tissue. Levels of vitamin C, an acutely reactive antioxidant, measured by high-performance liquid chromatography were not influenced by the CCl_4 injection in the cancer tissue whereas a significant decrease was evident in normal livers. The total fatty acid content, measured by gas chromatography, was significantly lower in the cancer tissue than in the normal liver while the ratio of polyunsaturated fatty acids (PUFAs) in total fatty acids was little changed. Resistance of hepatocellular cancer cells to fatty metamorphosis and their susceptibility to necrosis induced by free radicals may be due to the paucity of the target PUFAs in their cell membrane fraction, resulting in low levels of lipid peroxides. Peroxidation of PUFAs might act as a "shock absorber" against free radical-induced toxic cell death in normal cells.

Key words: Lipid peroxidation — Hepatocellular carcinoma — Polyunsaturated fatty acid — Vitamin C — Carbon tetrachloride

In cellular metabolism, oxygen radicals and resultant lipid peroxides provoke a variety of acute and chronic types of cell injury, such as dysfunction of the cell membranes, fatty metamorphosis, cell aging and cell death.[1–5] It is known that polyunsaturated fatty acids (PUFAs) in membrane lipids are especially susceptible to free radical-initiated peroxidation.[6] The oxidative DNA damage is caused not only by free radicals but also by lipid peroxides that finally propagate radicals and lead to the release of toxic breakdown products.[7,8] Lipid peroxidation is thought to play a key role in the "membrane-mediated chromosomal damage."[9] In this sense, a causal relationship between lipid peroxidation and carcinogenesis, particularly in its promotion phase, has been suggested.[10,11] Lowered lipid peroxidation in cancer cells, however, has also been emphasized by several investigators[12–20] in relation to changes in the fatty acid composition,[12,21,22] levels of antioxidants such as vitamin E[12,21] and reduced form of glutathione,[23–25] and enzyme activities of lipid peroxide-scavenging enzymes, including glutathione peroxidase (GSH-PO),[25–29] catalase,[25,30–33] superoxide dismutase[26,34] and glutathione *S*-transferases.[35–37]

On the basis of a series of experimental studies, we have hypothesized that lipid peroxides function as an intracellular "shock absorber" or "detour" to avoid free radical-induced cell injury.[38] It has been shown that expression of GSH-PO, a lipid peroxide-scavenger actively functioning in both the cytosol and mitochondria of normal hepatocytes,[38–40] is suppressed in 3′-methyl-4-dimethylaminoazobenzene (3′-methyl-DAB)-induced rat hepatocellular carcinoma.[29] GSH-PO synthesis is enhanced in cultured macrophages by experimentally induced lipid peroxidation.[41] The suppressed expression of GSH-PO in cancer may thus be related to the low amount of lipid peroxides within the cell. It is well known that cancer cells are far more susceptible to irradiation and free radical-releasing anticancer drugs such as adriamycin[4,42] than normal cells. In this context, the present study was specifically designed to investigate the resistance or susceptibility of carcinogen-induced hepato-

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cellular carcinoma tissues to lipid peroxidation. Cancer-bearing old rats were treated with a high dose of carbon tetrachloride (CCl_4), a strong lipoperoxidation inducer.[43,44] Patterns of fat staining, thiobarbituric acid (TBA) values, vitamin C (ascorbic acid) levels and fatty acid composition in *in vivo* tumor tissues were compared with those in normal livers. The cancer tissue exhibited resistance to lipoperoxidation-induced fatty metamorphosis, but was susceptible to free radical-induced cell death. Such a difference in the cellular response to CCl_4 treatment could be related to the relative paucity of PUFAs in the cell membrane fraction of the cancer tissue.

MATERIALS AND METHODS

Animals and diet Male Wistar rats weighing 80–100 g (4 weeks old) (n=90) were purchased from Imamichi Institute Corporation for Animal Reproduction, Omiya, and fed a basal diet (DE-2, Clea Japan, Tokyo) containing 0.06% 3′-methyl-DAB (Tokyo Chemical, Tokyo) for 16 weeks, followed by feeding the basal diet for 2 weeks, after which the animals were killed (at the 22nd week after birth). The control group (n=30) was fed the basal diet throughout the entire experimental period.

Induction of fatty liver Fatty liver was experimentally induced by subcutaneously injecting a high dose of CCl_4 (0.8 ml of 50% CCl_4 in corn oil per 100 g body weight), and the rats were killed 72 h later (n= 15 for rats fed the basal diet only and n=45 for rats fed the 3′-methyl-DAB-containing diet). Preliminary experiments revealed relative resistance of old rats to CCl_4-induced cell injury, and so the above experimental scheme was designed. In the aged rats, fatty change of the liver was mild and the TBA values were little changed after administration of the commonly employed dose of CCl_4 (0.2 ml of 50% emulsion per 100 g body weight). The time-course study using the control rats disclosed the maximal changes in morphology and TBA values 72 h after subcutaneous injection of the toxin.[45]

Tissue preparation Under ether anesthesia, arterial blood was collected from the thoracic aorta. Rats were then perfused with 0.01 *M* phosphate-buffered saline (PBS), pH 7.4, at room temperature for 2 min. Parts of the liver and tumor nodules were removed. Areas with grossly visible necrosis were carefully discarded. Histology of the tumors utilized for the study was confirmed by staining frozen sections with hematoxylin and eosin (HE). Further investigations were performed using histologically confirmed hepatocellular carcinomas. Cholangiocellular carcinomas and mesenchymal tumors were excluded from the study. The remaining liver tissues were routinely fixed in 10% formalin and examined histologically by preparing paraffin-embedded sections.

Fat stain Oil red O staining was performed on periodate-lysine-4% paraformaldehyde (PLP)-fixed frozen sections in a routine manner.

Biochemical assay Lipid peroxide levels in the liver and sera were measured by the TBA method using a fluorescence spectrophotometer (model MPF4; Hitachi, Tokyo), as described by Yagi.[46] The samples were kept frozen at −20°C until analysis. The liver and tumor tissues were homogenized at a dilution of 1:10 in 0.01 *M* Tris-hydrochloric acid buffer, pH 7.6, containing 5 m*M* ethylenediamine tetraacetic acid. For assaying fatty acids and vitamin C, the liver and tumor tissues (n=6 in each group) were homogenized at a dilution of 1:20 in a 1:2 mixture of chloroform and methanol. The supernatants were collected by centrifugation at 12,000 rpm for 10 minutes. The fatty acid composition was determined after May and McCay[47] using a gas chromatography detection system (model GC-4CM; Shimadzu, Kyoto). Levels of vitamin C in tissues were measured by high-performance liquid chromatography (HPLC) using an HPLC pump (model 880-PU; Japan Spectroscopic Co., Tokyo).[48] The data were analyzed using a computer system (model Chromatopac C-R4A; Shimadzu).

RESULTS

Incidence of hepatocellular carcinoma Of 90 rats fed 3′-methyl-DAB, about one-third (n=32) developed foci of hepatocellular carcinoma that were large enough for the following studies. The diameter of the tumors was up to 1 cm. Almost all of the rat livers bore cholangiocellular carcinoma and microscopic hyperplastic foci. Some individuals developed mesenchymal liver tumors. These non-hepatocellular tumor tissues were excluded from the study.

Histology After CCl_4 injection, severe fatty metamorphosis occurred in nonneoplastic hepatocytes of both the experimental and control rats, as clearly shown by oil red O staining (Fig. 1). The fatty change was prominent in the centrilobular zone of the liver. In contrast, hepatocellular carcinoma cells scarcely showed fatty metamorphosis (Figs. 1 and 2). Marked fatty change was not seen in the viable cancer cells. However, the CCl_4 administration often provoked spotty as well as massive necrosis of the tumor cells (Fig. 2). Ballooning degeneration of the cancer cells was also frequently observed. Spotty necrosis and ballooning were inconspicuous in the nonneoplastic hepatocytes of CCl_4-treated rats, and massive necrosis was never observed. Hepatocellular carcinomas without CCl_4 treatment did not show massive necrosis, and spotty necrosis and ballooning were mild, if any.

Lipid peroxidation assay Lipid peroxide levels, expressed as levels of TBA-reactive substances, in the liver and sera

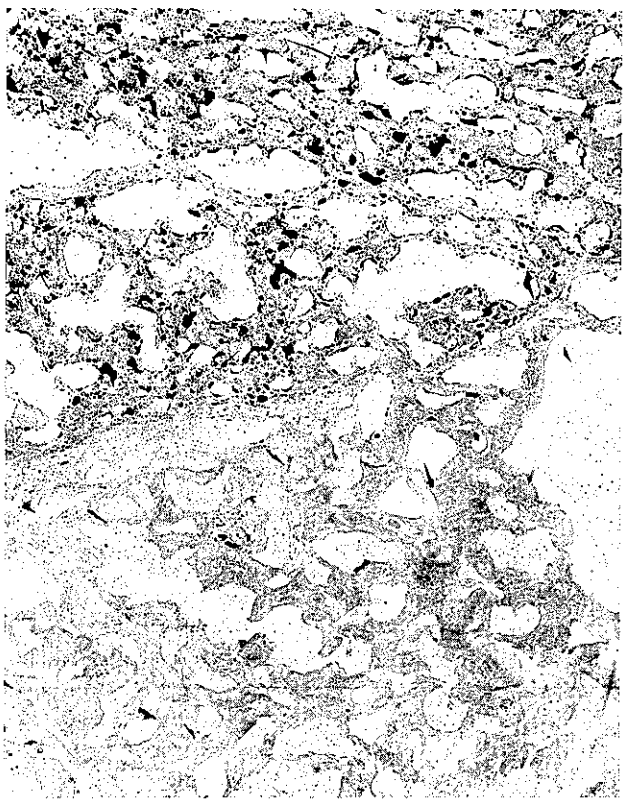

Fig. 1. Oil red O staining in hepatocellular carcinoma and the surrounding liver tissue 72 h after CCl_4 treatment. The cancer cells (lower half) show minimal fatty change whereas the adjacent hepatocytes (upper half) contain a good number of fat globules in the cytoplasm. Sinusoids were artificially dilated by perfusing PBS before sampling. (×200, frozen section)

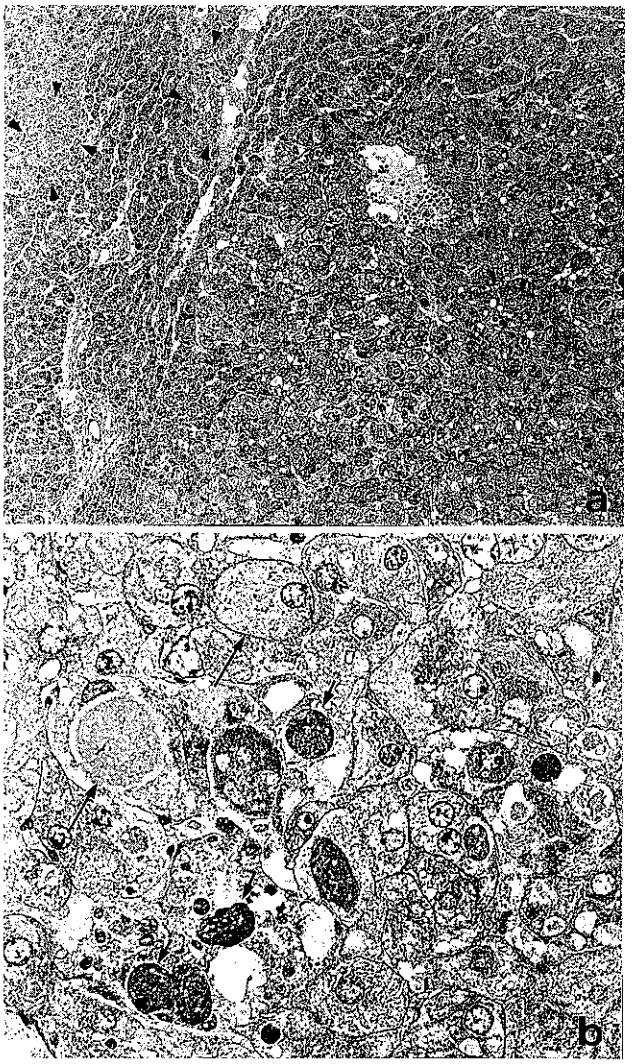

Fig. 2. Histologic alterations of hepatocellular carcinoma (right half of Fig. 2a) and adjacent nonneoplastic liver tissue (left half) 72 h after CCl_4 administration. The nonneoplastic hepatocytes show evident fatty metamorphosis (arrowheads) (a). In contrast, coagulation necrosis (short arrows) and ballooning (long arrows) are apparent in the cancer cells (b). (a: HE, ×75; b: HE, ×300, paraffin-embedded sections)

are summarized in Table I. In the control liver, accompanying the severe fatty metamorphosis induced by CCl_4 injection, the TBA values increased to twice those in the untreated liver ($P<0.01$). In hepatocellular carcinoma, in which fatty change was rare, the TBA values were not significantly altered by the CCl_4 treatment. Basal TBA values in the cancer tissue in rats without CCl_4 treatment were not statistically different from those in the normal liver. In the sera, the TBA values before and after CCl_4 treatment did not show any significant difference.

Vitamin C levels The results are summarized in Table II. Basal levels of vitamin C in hepatocellular carcinoma tended to be higher than those in the normal control liver, but the difference between these two groups was not significant. In the normal liver, the vitamin C content showed a considerable decrease after CCl_4 treatment ($P<0.05$). Vitamin C levels in the cancer tissue exhibited a considerable variation from tumor to tumor, and the change induced by CCl_4 treatment was not statistically significant.

Fatty acid composition The results are shown in Table III. The total fatty acid content in the membrane fraction of hepatocellular carcinoma was low when compared with that in the normal liver. The difference between the normal liver and cancer was statistically significant ($P<0.05$). While the fatty acid composition or the ratio of

Table I. Thiobarbituric Acid (TBA) Values (Representing Levels of Lipid Peroxides) in Rat Liver and Sera before and after CCl_4 Treatment

Animal group	CCl_4 injection	Liver TBA-reactive substances (nmol/g) Average±standard deviation (−)	(+)	Serum TBA-reactive substances (nmol/g) Average±standard deviation (−)	(+)
Normal control rat (n=9)		89.9±15.7	179.6±38.4	4.13±0.41	4.35±0.60
			$P<0.01$*		
Cancer-bearing rat (n=15)		106.4±32.8[a]	113.7±41.2[a]	4.22±0.80	4.19±0.40

a) Hepatocellular carcinoma tissues were sampled for measurement.
* After CCl_4 treatment, the TBA values were significantly ($P<0.01$) increased in the control liver. However, this treatment did not influence the TBA values in hepatocellular carcinomas or in sera. No statistically significant difference was found between the TBA values in normal livers and those in hepatocellular carcinomas.

Table II. Vitamin C Contents in Normal Liver and Hepatocellular Carcinoma before and after CCl_4 Treatment

Animal group	Vitamin C content (μmol/g wet tissue) Average±standard deviation	
CCl_4 treatment	(−)	(+)
Normal liver (n=6)	0.65±0.11	0.48±0.12
	$P<0.05$	
Hepatocellular carcinoma (n=6)	0.97±0.42	0.81±0.36

saturated versus unsaturated fatty acids was not significantly different between the normal and cancerous tissues, the absolute amount of PUFAs was evidently lower in hepatocellular carcinoma than in the normal liver.

DISCUSSION

Malignant tumors are known to be highly susceptible to free radicals generated by irradiation and anticancer reagents such as adriamycin.[4, 42] The present study was designed to analyze the mechanisms of such radical-induced injury in cancer cells. Rats bearing carcinogen-induced hepatocellular carcinoma were treated with a high dose of CCl_4, one of the most drastic radical inducers.[43, 44] The lack of change in patterns of fat staining, TBA values and vitamin C levels after CCl_4 insult clearly indicated that lipid peroxidation was hardly induced in hepatocellular carcinoma. The nonneoplastic hepatocytes showed high susceptibility to the same treatment, as demonstrated by formation of marked fatty metamorphosis with a concomitant increase in lipid peroxide levels.[45] The cancer cells often became degenerative and necrotic after CCl_4 injection, but such changes were scarcely observed in the nonneoplastic hepatocytes under

Table III. Contents of Total Membrane Lipids and Fatty Acid Composition in Normal Rat Liver and Hepatocellular Carcinoma

Measurement	Average±standard deviation Normal control liver (n=6)	Hepatocellular carcinoma (n=6)
Total membrane lipids nmol/5 mg wet tissue	4.07±1.86	1.49±0.55
	$P<0.05$	
Fatty acid composition (%)[a]		
16:0 (palmitic acid)	26.3±1.2%	22.1±4.4%
18:0 (stearic acid)	20.6±4.6%	16.8±3.0%
18:1 (oleic acid)	12.7±2.6%	20.1±5.7%
18:2 (linoleic acid)	18.2±2.4%	19.7±4.1%
20:4 (arachidonic acid)	21.9±1.7%	18.6±5.0%

a) The percent composition of unsaturated fatty acids was not significantly different between the normal liver and hepatocellular carcinoma.

the present experimental protocol. It is suggested that free radicals caused necrosis of the cancer cells by lipid peroxidation-independent mechanisms. In other words, the process of lipid peroxide formation may function as a "shock absorber" or "detour"[38] in the normal hepatocytes during the process of free radical-induced cell injury.[3,4] Water-compatible free radicals can react with DNA, enzymes and other biologically active substances[7,8] instantly and directly, as compared to lipid peroxide molecules. Moreover, the latter have a native scavenger, GSH-PO, in many cells to detoxify them.[38-40]

A good number of studies have shown that lipid peroxidation is significantly decreased in rat tumor cells as compared with the corresponding normal counterpart.[12-20] However, most of these studies employed cultured tumor cells as a tumor source, and the comparison was often made between the cultured cells *in vitro*, particularly Novikoff tumor cells, and normal liver tissue *in vivo*.[12,13,19] Chemical and cell biologic characteristics of the membranes, as well as the susceptibility to peroxidation or other toxic processes, of cultured cells may differ from those of the *in vivo* counterpart. Moreover, Novikoff tumor cells themselves may well be cholangiogenic in origin.[12,13,19]

The present study clearly showed decreased levels of lipid peroxidation in hepatocellular carcinoma *in vivo* as compared with that in the normal liver. We intended to compare changes in the cancer tissue with those in the normal liver in the control group, instead of comparing with those in the noncancerous portions of the cancer-bearing liver. This is because such noncancerous portions often bore microscopic hyperplastic foci in which metabolic changes similar to those seen in cancer cells have been demonstrated.[20,23,26,28,30-33,35-37,49] The lowered response of cancer cells to the lipoperoxidative insult can be explained by the relative paucity of PUFAs, the target or substrate of lipid peroxidation, in the cell membrane fraction of cancer cells,[6,12,21] as was confirmed in the present study. It is intriguing to hypothesize that such paucity of PUFAs in tumor tissues is related to their low content in actively proliferating normal tissues.[12,19,22] It has been shown that the levels of cytochrome P-450, a microsomal drug-metabolizing enzyme which catalyzes formation of free radicals such as CCl_3 radicals and oxygen radicals, are maintained in tumor tissues,[6,12,50] although a decreased level of this enzyme has also been documented.[51] We have shown that immunoreactivity of cytochrome P-450 is detectable in preneoplastic hyperplastic foci in the 3′-methyl-DAB-treated rat liver.[52] The evidence signifies that cancer cells maintain the radical-generating system intact.

Changes in antioxidant status have been reported repeatedly. Levels of vitamin E (α-tocopherol) and total activities of antioxidants are substantially increased in cultured Novikoff tumor cells when compared with the normal liver, and the increased levels of vitamin E in the membrane fraction are thought to offer quite effective protection against lipid peroxidation under such experimental conditions.[12,21] However, there has been no detailed report on the levels of vitamin E in *in vivo* tumor tissues. In contrast, tissue levels of reduced form of glutathione, a representative water-soluble antioxidant, are significantly lower in the cancer tissue than in the normal counterpart.[23-25] Oral administration of reduced glutathione results in the regression of aflatoxin B1-induced hepatocellular carcinoma in the late stage of tumor progression.[53,54] Similarly, vitamin C, another water-soluble antioxidant, allegedly exhibits inhibitory effects against growth of sarcoma 180 in mice[55] and human tumors *in vitro*,[56] although conflicting findings have also been presented.[57] In the present study, the level of vitamin C in the untreated cancer tissue was comparable to or even higher than that in the normal liver. This indicates that the capacity of vitamin C synthesis and/or uptake by hepatocellular carcinoma cells is as active as that seen in their normal counterpart.[58]

Among peroxide-scavenger enzymes, lowered activities of catalase,[25,30-33] superoxide dismutase[26,34] and GSH-PO[26] in tumor tissues, including human hepatocellular carcinoma,[25,27] have been described. In the rat liver, low levels of GSH-PO have been observed not only in carcinogen-induced hepatocellular carcinoma[29,52] but also in preneoplastic hyperplastic foci.[28,49,52] Glutathione reductase activity and immunoreactivity are conversely increased in such preneoplastic and neoplastic lesions.[25,27,49,52] The placental type (pi) of glutathione *S*-transferase, a representative detoxification enzyme group catalyzing glutathione conjugation, is specifically expressed in these hepatic lesions.[35] GSH-PO-like activity of the pi isozyme, however, is negligible.[59,60] Total enzyme activities of glutathione *S*-transferases, encompassing alpha, mu and pi isozyme groups,[59] are unaffected during the course of carcinogenesis.[37] Consequently, the reduction of lipid peroxides in the cancer tissue could not be explained solely by such changes in the lipid peroxide-scavenging enzymes.

In conclusion, the phenomena seen in experimentally induced hepatocellular carcinoma during the course of CCl_4 toxicity can be attributed to the decrease in the content of PUFAs in the membrane compartments. Changes in vitamin E levels in cancer cells should further be investigated by *in vivo* experiments. Reduced levels of water-soluble antioxidants and GSH-PO in the tumor tissue might be secondary to such membrane alterations. Expression of GSH-PO, for example, has been shown to increase in cultured cells in the presence of lipid peroxides.[41] The relative paucity of lipid peroxides may result in the reduction of GSH-PO synthesis.

Further studies are required to clarify the relationship between the promoting effect of lipid peroxides shown in an early phase of carcinogenesis and the low content of lipid peroxides observed in developed cancer tissues.

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