

Development of Hepatocellular Adenomas and Carcinomas Associated with Fibrosis in C57BL/6J Male Mice Given a Choline-deficient, L-Amino Acid-defined Diet

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Development of hepatocellular carcinomas in rats caused by a choline-deficient, L-amino acid-defined (CDAA) diet, usually associated with fatty liver, fibrosis, cirrhosis and oxidative DNA damage, has been recognized as a useful model of hepatocarcinogenesis caused by endogenous factors. In the present study, in order to further explore involved factors and genes, we established an equivalent model in spontaneous liver tumor-resistant C57BL/6J mice. Six-week-old males and females were continuously fed the CDAA diet and histological liver lesions and oxidative DNA damage due to 8-hydroxydeoxyguanosine (8-OHdG) were examined after 22, 65 and 84 weeks. In male mice, fatty change and fibrosis were evident at 22 weeks, and preneoplastic foci of altered hepatocytes were seen at an incidence of 8/8 (100%) and a multiplicity of 6.6 ± 4.0 per mouse at 65 weeks. Hepatocellular adenomas and carcinomas developed at incidences of 16/24 (66.7%) and 5/24 (20.8%), and multiplicities of 1.42 ± 1.32 and 0.29 ± 0.62 , respectively, at 84 weeks. The female mice exhibited resistance to development of these lesions. The CDAA diet also increased 8-OHdG levels in male but not female mice. These results indicate that a CDAA diet causes hepatocellular preneoplastic foci, adenomas and carcinomas associated with fibrosis and oxidative DNA damage in mice, as in rats, providing a hepatocarcinogenesis model caused by endogenous factors in mice.

Key words: C57BL/6J male mice — Choline-deficiency — Hepatocarcinogenesis

Descriptions of the variety of pathological lesions caused by lipotrope-deficient diets lacking choline, methionine, folic acid or vitamin B₁₂, all involved in the generation of labile methyl groups, date back to the 1930s–1950s, when Best and Huntsman first discovered choline to be a dietary factor that can prevent and help repair fatty liver.^{1,2)} The underlying physiology is still of great interest, and the fact that prolonged feeding of a choline-deficient, methionine-low (CDML) diet causes hepatocellular carcinomas (HCCs), usually associated with fatty change, hepatocyte injury, fibrosis and cirrhosis and induction of oxidative DNA damage in rats, has attracted particular attention.^{3–5)} We have further found a choline-deficient, L-amino acid-defined (CDAA) diet to possess a greater capacity than a casein-based semisynthetic CDML diet to cause such lesions in rats,⁶⁾ and have focused on its use in an experimental model of hepatocarcinogenesis caused by endogenous factors, with similarities in the histopathological sequence to human hepatocellular carcinoma development accompanying cirrhosis.⁷⁾ So far, repeated cycles of hepatocyte injury and regeneration,⁸⁾ inhibition of apoptosis,⁹⁾ oxidative stress,^{5, 10, 11)} hypomethylation of RNAs and DNA including the 5′-upstream region of the *c-myc* gene,^{12, 13)} chronic activation of protein kinase C¹⁴⁾ and activation of the arachidonic acid cascade^{15, 16)} have been pos-

tulated to be involved. Major roles for gene mutations in *Ki-ras*, *p53*, *p16*, *p21* and *β-catenin*, however, appear unlikely.^{17, 18)} Recently, we have further found a possible involvement of up-regulated expression of cyclooxygenase (COX)-2,¹⁹⁾ but details of the underlying mechanisms largely remain to be elucidated.

Recent studies using genetically modified, transgenic, gene-disrupted, or mutated mice have provided profound information on the biological functions of various genes and their involvement in different physiological and pathological phenomena, including carcinogenesis.^{20–22)} Studies of hepatocarcinogenesis caused by a CDML diet in mice have been limited to investigations with closed colonies in the 1950s^{23, 24)} or with spontaneous liver tumor-susceptible C3H and B6C3F1 mice.^{25–27)} In the present study, in order to establish a hepatocarcinogenesis model caused by endogenous factors in mice, the hepatocarcinogenicity of a CDAA diet, together with the induction of 8-hydroxydeoxyguanosine (8-OHdG), a marker of oxidative DNA damage, was examined in the spontaneous liver tumor-resistant C57BL/6J strain.²⁸⁾

MATERIALS AND METHODS

Chemicals An authentic sample of 8-OHdG was purchased from Wako Pure Chemical Industries, Ltd., Osaka.

Animals and diets Five-week-old C57BL/6J male and

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female mice, 70 each, were obtained from Japan Clea, Osaka, and housed 5 to 6 in plastic cages with soft wood chips as bedding. The room temperature was kept at 23±2°C and the humidity at 55±10%, with a 12-h light/dark cycle. The CDAA diet (No. 518753) and the control choline supplemented, L-amino acid-defined (CSAA) diet (No. 518754) were purchased from Dyets Inc., Bethlehem, PA. Details of the composition and the lipotrope contents of the CDAA and CSAA diets have been described previously.^{6,15} The diets were stocked at 4°C in the dark, and given to the animals *ad libitum* by freshly replenishing feed trays weekly, when food consumption and body weights were measured.

Experimental protocol The male and female mice, 6 weeks old at the commencement of the experiment, were divided into two groups. Group 1 was continuously fed the CDAA diet, and group 2 the control CSAA diet, animals being sacrificed under ether anesthesia 22, 65 and 84 weeks after the commencement of the experiment. The livers were excised and subjected to histological analyses for preneoplastic and neoplastic lesions and fibrosis. Portions were frozen in liquid nitrogen and subjected to biochemical analysis of 8-OHdG. The kidneys, spleen, lung, thyroid, thymus, pancreas, salivary gland, brain and macroscopically discernible tumors were excised, weighed and histologically examined.

Histological analysis A single slice from each liver lobe for the 22- and 65-week point analyses, and every other

slice from entire livers step-sliced at 3 mm thickness for the 84-week point analysis, were fixed in 10% buffered neutral formalin, routinely processed for paraffin embedding, sectioned and subjected to either hematoxylin and eosin (HE) or azan-Mallory staining. Preneoplastic and neoplastic liver lesions were diagnosed according to the criteria of Frith and Ward²⁹ and fibrosis was assessed from the degree of azan-Mallory staining.

Measurement of 8-OHdG levels DNA was extracted from frozen liver tissue using a DNA Extractor WB Kit (Wako Pure Chemical Industries, Ltd.) and hydrolyzed into nucleosides as described previously,^{15,16} but in the presence of 0.1 mM deferoxamine mesylate (Sigma Chemical Co., St. Louis, MO).³⁰ 8-OHdG was determined, basically according to the method of Kasai, using an HPLC system connected to an electrochemical detector Coulochem II (ESA Inc., Bedford, MA) and eluted with 10 mM NaH₂PO₄ solution containing 5% methanol, as detailed previously.^{15,16}

RESULTS

Development of fatty change, fibrosis and altered foci in the livers of mice on CDAA diet for 22 or 65 weeks

Final body and liver weights, diet intake and histological liver findings for male and female mice maintained on the CDAA diet for 22 and 65 weeks are summarized in Table I. The liver weights of males but not females were signifi-

Table I. Body and Liver Weights, Diet Intake and Histological Liver Findings for Mice Given a CDAA Diet for 22 or 65 Weeks^{a)}

Groups	No. of mice	Final body wt. (g)	Liver wt. (g) (ratio to body wt.)×10 ⁻²	Diet intake (g/day/kg body wt.)	Histological liver findings					Fatty change	Fibrosis			
					Altered foci			Incidence (%)	Multi-plicity ^{b)} (No./mouse)			Histological types (%)		
					Total No. counted	Acidophilic	Clear cell							
Males														
22 weeks														
CDAA diet	7	36.1±5.4	2.27±0.6 ^{c)} (6.21±0.75) ^{c)}	106±14						++~+++	++			
CSAA diet	2	38.8±6.4	1.78±0.6 (4.53±0.76)	88±20						±~+	-			
65 weeks														
CDAA diet	8	38.4±8.4	2.23±0.7 ^{c)} (5.75±0.74) ^{c)}	90±18	8 (100) ^{c)}	6.6±4.0	53 (100)	49 (92.5)	4 (7.5)	+++~++++	+++~++++			
CSAA diet	2	39.0±0.5	1.71±0.1 (4.38±0.09)	84±14	0	0				±~++	-			
Females														
22 weeks														
CDAA diet	7	28.2±2.8	1.52±0.2 (5.37±0.31)	128±17						+~+++	+			
CSAA diet	2	26.3±0.9	1.45±0.5 (5.51±0.19)	143±22						-	-			
65 weeks														
CDAA diet	9	32.7±9.2	1.99±0.6 (6.23±1.63)	119±14	1 (11.1)	0.1±0.3	1 (100)	1 (100)	0	++~+++	++			
CSAA diet	3	28.5±13.4	1.66±0.7 (5.90±0.31)	151±40	0	0				-~+	-			

a) Values are mean±SD.

b) Single slice analyzed per lobe.

c) Significantly different from the respective CSAA diet group value (P<0.05).

cantly increased, in terms of both absolute and ratio to body weight values, compared with the respective control groups receiving the CSAA diet.

Macroscopically, the livers of male but not female mice on a CDAA diet for 22 weeks exhibited slight features of fibrosis (data not shown). Those of males after 65 weeks, as shown in Fig. 1A, demonstrated multiple small whitish spots. Histologically, fatty change and fibrosis were observed in both male and female mice, but to a much lesser extent in the latter, after 22 weeks (Fig. 1, C and D) on the CDAA diet, with a further advance after 65 weeks (Fig. 1, E and F).

Preneoplastic foci of phenotypically altered hepatocytes developed in male livers on a CDAA diet for 65 weeks, at an incidence of 100% and a multiplicity of 6.6 ± 4.0 per mouse (one slice from each lobe examined), and at 11.1% and 0.1 ± 0.3 in the females. The majority of the foci proved to be of acidophilic (eosinophilic) type with a ground-glass appearance, consisting of either relatively enlarged (27 out of 49 counted, 55.1%) (Fig. 1G) or smaller hepatocytes (22/49, 44.9%) as compared to the background parenchyma. Clear cell foci accounted for only 4 out of 53 lesions (7.5%) in the males and none in

the females. Basophilic foci were not observed. At the 22-week time point, foci of altered hepatocytes were not discernible among the severe fatty changes evident on HE staining.

Development of hepatocellular adenomas and carcinomas in the livers of mice on CDAA diet for 84 weeks

Data for final body and liver weights, diet intake and liver tumors developing in male and female mice maintained on a CDAA diet for 84 weeks are summarized in Table II. The liver weights of males and females on the CDAA diet were significantly increased in terms of both absolute and ratio to body weight values compared with the control mice on a CSAA diet.

Macroscopically, the male livers exhibited fibrosis but not cirrhosis, with multiple whitish or brownish protuberant nodules (Fig. 2, A and B). Those of females showed slight fibrotic features (data not shown). Histologically, fatty change and fibrosis were further advanced at the 84-week time point in both males and females, but again to a lesser extent in the latter. Hepatocellular adenomas compressing the surroundings developed in the males at an incidence of 66.7% and a multiplicity of 1.42 ± 1.32 per mouse. The majority (32 out of 34 counted) was acido-

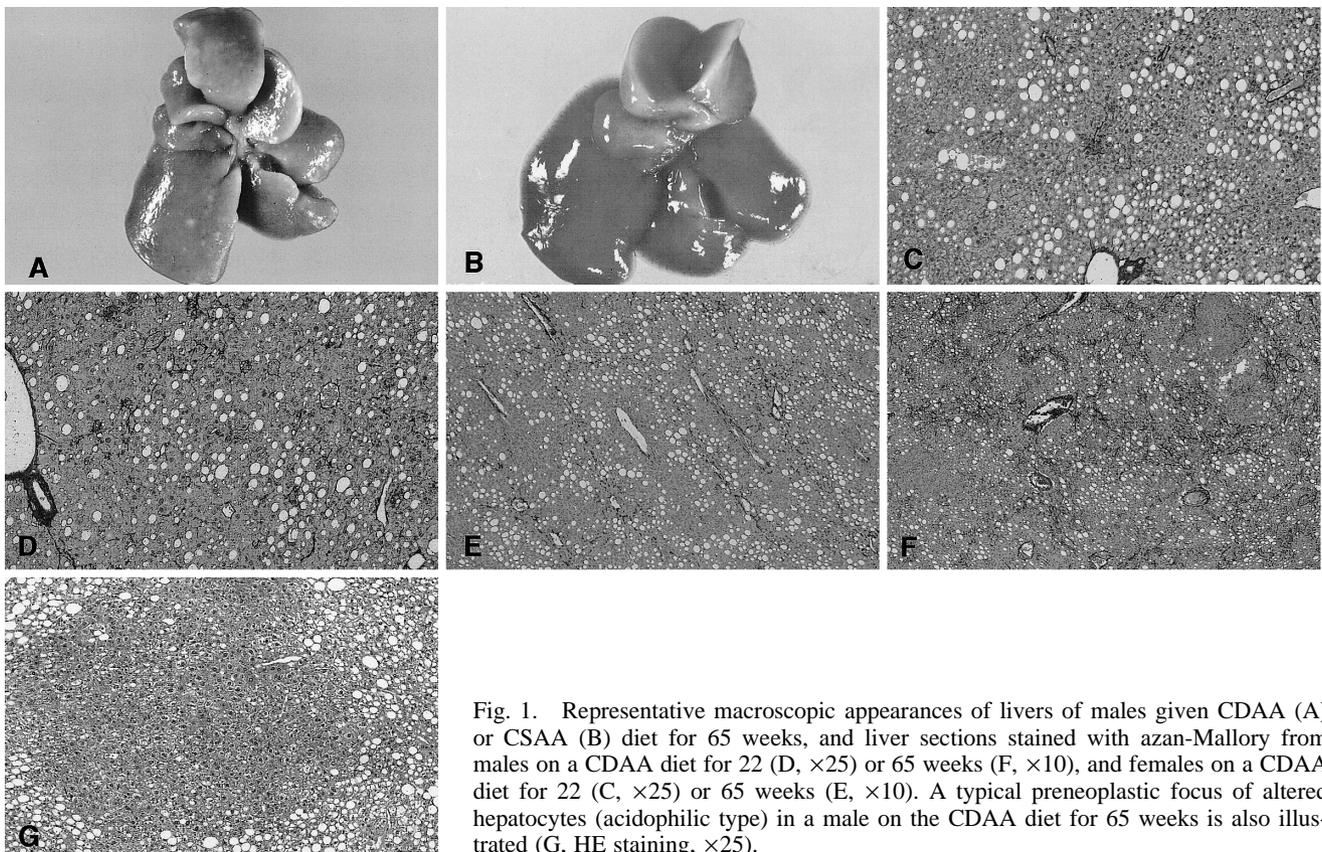


Fig. 1. Representative macroscopic appearances of livers of males given CDAA (A) or CSAA (B) diet for 65 weeks, and liver sections stained with azan-Mallory from males on a CDAA diet for 22 (D, $\times 25$) or 65 weeks (F, $\times 10$), and females on a CDAA diet for 22 (C, $\times 25$) or 65 weeks (E, $\times 10$). A typical preneoplastic focus of altered hepatocytes (acidophilic type) in a male on the CDAA diet for 65 weeks is also illustrated (G, HE staining, $\times 25$).

Table II. Body and Liver Weights, Diet Intake and Incidence and Multiplicity Data for Liver Tumors Developed in Mice Given a CDAA Diet for 84 Weeks^{a)}

Groups	Effective No. of mice	Final body wt. (g)	Liver wt. (g) (ratio to body wt.) ×10 ⁻²	Diet intake (g/day/kg body wt.)	Liver tumors ^{b)}								
					Incidence (%)				Multiplicity (No. per mouse)				
					AD	HCC	HM	HMS	AD	HCC	HM	HMS	
Males													
CDAA diet	24	38.0±7.5	2.85±0.8 ^{c)} (7.74±2.81) ^{d)}	84±15	16 ^{e)} (66.7)	5 (20.8)	5 (20.8)	1 (4.2)	1.42 ^{e)} ±1.32	0.29 ±0.62	0.21 ±0.42	0.04 ±0.20	
CSAA diet	12	37.3±7.4	1.62±0.4 (4.35±0.51)	85±11	1 (8.3)	0	0	0	0.08 ±0.29	0	0	0	
Females													
CDAA diet	19	34.2±7.3 ^{f)}	2.40±0.5 ^{c)} (7.23±1.77) ^{e)}	110±13	1 (5.3)	0	1 (5.3)	0	0.05 ±0.23	0	0.05 ±0.23	0	
CSAA diet	7	27.5±6.7	1.48±0.3 (5.43±0.38)	113±22	0	0	0	0	0	0	0	0	

a) Values are mean±SD.

b) AD, hepatocellular adenoma; HCC, hepatocellular carcinoma; HM, hemangioma; HMS, hemangiosarcoma.

c) Significantly different from the respective CSAA diet group value $P < 0.0001$.

d) Significantly different from the respective CSAA diet group value $P < 0.0002$.

e) Significantly different from the respective CSAA diet group value $P < 0.002$.

f) Significantly different from the respective CSAA diet group value $P < 0.05$.

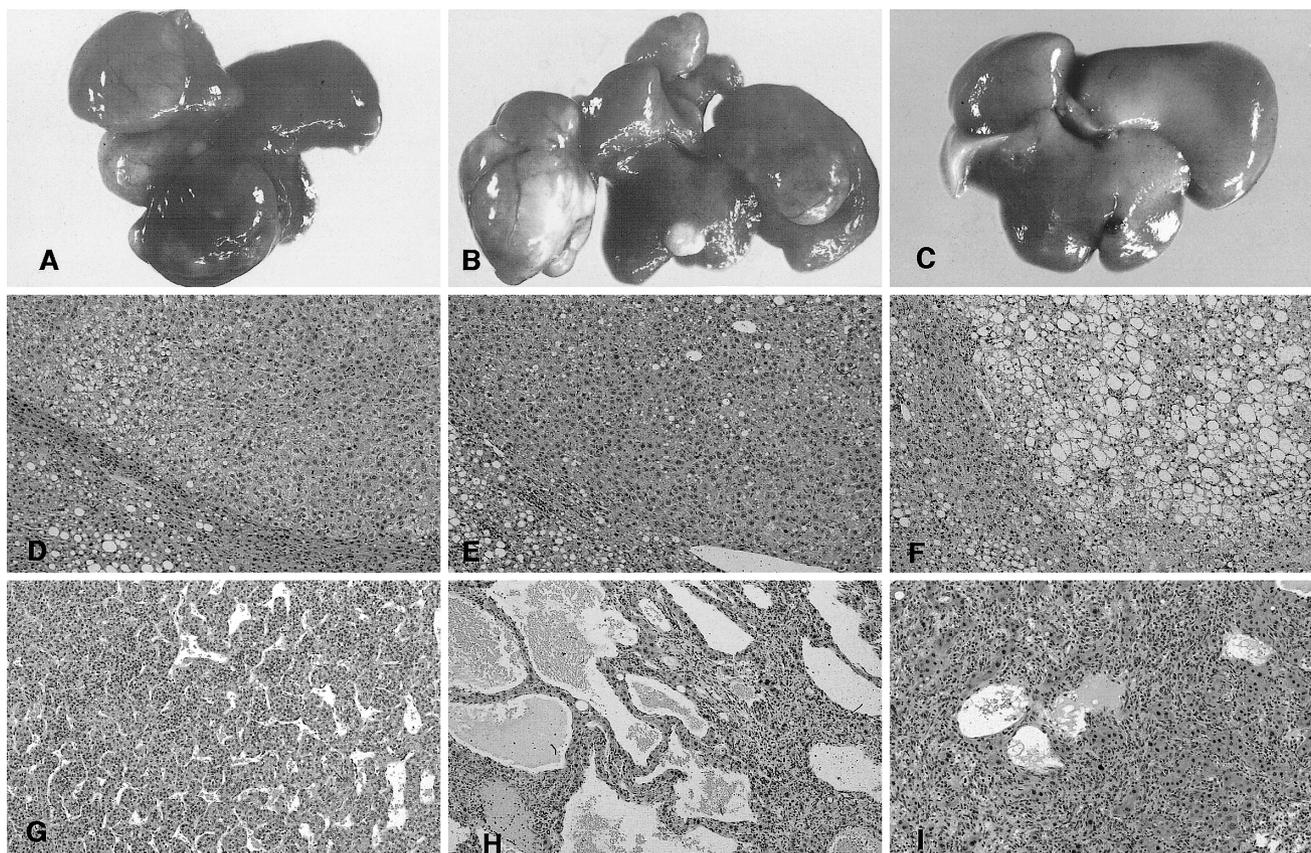


Fig. 2. Representative macroscopic appearance of livers of males given CDAA (A, B) or CSAA (C) diet for 84 weeks, and histological findings for HE-stained features of hepatocellular adenomas (D, E, acidophilic type; F, vacuolated cell type, ×25), a HCC (G, ×33), a hemangioma (H, ×25), and a hemangiosarcoma (I, ×33).

philic (eosinophilic) type with a ground-glass appearance, having relatively large nuclei and prominent nucleoli (Fig. 2, D and E). Vacuolated cell adenomas (Fig. 2F) were rarely observed (2 out of 34). In the females, foci of altered hepatocytes, all of acidophilic type with a ground-glass appearance, were observed at an incidence of 10/19 (52.6%) and a multiplicity of 0.63 ± 0.68 , but only one adenoma of acidophilic type was observed. Hepatocellular carcinomas, all well differentiated with trabecular (Fig. 2G) or infrequently adenomatous patterns, developed only in the males at an incidence of 5 out of 24 (20.8%) and a multiplicity of 0.29 ± 0.62 per mouse. Hemangiomas (Fig. 2H) were observed in 5/24 (20.8%) males with a multiplicity of 0.21 ± 0.42 , and in only one of the females. A hemangiosarcoma (Fig. 2I) with metastasis to the mesenteric lymphnodes developed in one male.

8-OHdG levels in the liver DNA The 8-OHdG levels in the liver DNA of male and female mice on the CDAA diet for 22 or 65 weeks are summarized in Table III. The values of males and females on the CSAA diet for 22 or 84 weeks were combined because of the small numbers of animals in the CSAA diet groups and the absence of significant differences among their values. 8-OHdG levels in males on the CDAA diet were significantly increased at the 65-week time point, only tending to increase at the 22-week point, compared with the CSAA diet group values. Those for females showed no significant increase at any time point.

Tumors in other organs There were no significant differences in the weights of organs other than the liver, in terms of both absolute and ratio to body weight values, between the CDAA and CSAA groups of males or females at any time point (data not shown). No tumors were observed at the 22- or 65-week time point. Those observed after 84 weeks are summarized in Table IV. There were no significant differences in incidences between the two groups. Lymphomas were the most frequent tumors, those in males being histologically of small lymphocytic (3

mice) and large cleaved type follicular cell type (1 mouse), all developing in lymphnodes. Those in females were follicular center cell lymphomas, either mixed or of large cleaved type (one mouse each) in the spleen, and a plasmacytoma in a lymphnode. Bronchiolo-alveolar adenomas were observed in males of both CDAA and CSAA diet groups, one solid alveolar and one papillary bronchiolar type in the CDAA diet group, and four papillary type lesions in the CSAA diet group. Pituitary adenomas and thyroid follicular cell adenomas were also observed in females on the CDAA diet, and skin basal cell hyperplasias in a male on the CDAA diet and females on the CDAA or CSAA diets.

DISCUSSION

The present results clearly indicated that a CDAA diet causes preneoplastic foci of altered hepatocytes, and subsequently hepatocellular adenomas and HCCs associated with fatty change, fibrosis and induction of an oxidative

Table III. 8-OHdG Levels in the Liver DNA of Mice Given a CDAA Diet for 22 or 65 Weeks^{a)}

Groups	Experimental periods (weeks)	No. of mice examined	8-OHdG/10 ⁵ dG
CDAA diet			
Males	22	7	0.574±0.205
	65	6	1.088±0.540 ^{c)}
Females	22	4	0.498±0.034
	65	7	0.495±0.150
CSAA diet ^{b)}		22-84	7
(Males+Females)			0.487±0.129

a) Values are mean±SD.

b) Values for the males and females were combined.

c) Significantly different from the CSAA diet group value $P < 0.01$.

Table IV. Incidences of Tumors Developing in Other Organs of Mice Given a CDAA Diet for 84 Weeks

	Incidence (%)			
	Male mice		Female mice	
	CDAA	CSAA	CDAA	CSAA
Number of animals examined	24	12	19	7
Tumors				
Lymphoma	4 (16.7)	0	3 (15.8)	0
Lung bronchiolo-alveolar adenoma	2 (16.7)	3 (25.0)	0	0
Pituitary gland adenoma	0	0	2 (10.5)	0
Skin basal cell hyperplasia	1 (4.2)	0	2 (10.5)	1 (14.3)
Thyroid follicular cell adenoma	0	0	1 (5.3)	0

DNA damage 8-OHdG, in spontaneous liver tumor-resistant C57BL/6J male mice. Except for the lack of obvious cirrhosis, the lesions were basically similar to, if less pronounced than, those caused by a CDAA diet in Fischer 344 male rats. Earlier we reported the CDAA diet to be associated with severe fibrosis and enzyme-altered preneoplastic foci at 12 weeks, cirrhosis at 30 weeks, and hepatocellular adenomas and HCCs at the incidences of 100% at 52 weeks, in rats.^{5, 6, 15, 16, 19} The present findings of no discernible preneoplastic lesions at the 22-week point, might be due to the lack of a sensitive marker enzyme for very small foci in mice, such as glutathione S-transferase placental form in rats.^{31, 32} However, the results clearly demonstrated for the first time to the authors' knowledge, that a CDAA diet *per se* is hepatocarcinogenic in spontaneous liver tumor-resistant C57BL/6J mice, as in rats. Nevertheless, since the present study was focused on describing events that occur during hepatocarcinogenesis, the causes or triggers that induce oxidative DNA damage 8-OHdG and fibrosis, and their roles in the development of foci and tumors largely remain to be elucidated.

Reportedly, in spontaneous liver tumor-prone B6C3F1 males, even a casein-based CDML diet causes foci or nodules of altered hepatocytes at 3 months and HCCs in an incidence of 8% at 13 months.²⁷ The higher susceptibility than found for the present C57BL/6J mice might be ascribable to promotion of spontaneously initiated hepatocytes.²⁷ However, the liver tumor-prone C3H strain appears to be rather resistant, with hepatocellular adenomas but not HCCs developing in only 1/23 mice maintained on a CDAA diet for 52 weeks, presumably because of a relative resistance to DNA hypomethylation²⁶ or a high N²-guanine tRNA methyltransferase II activity in the liver.^{33, 34} Our present results, suggesting an intermediate susceptibility of the C57BL/6J strain between B6C3F1 and C3H, might thus provide a new insight into the hepatocarcinogenic mechanisms of the CDML diet.

Our results also point to a relative resistance of C57BL/6J females compared with males, which also holds true for rats.³⁵⁻³⁷ In humans, the development of cirrhosis, HCCs and HCCs in cirrhosis is known to be more frequent in men than women.^{38, 39} Sexual dimorphism in various functions of the liver is well known and the liver possesses

nuclear and cytosolic estrogen receptors.⁴⁰ Regarding mechanisms, phosphatidylcholine (PC) synthesis depends more on the methylation pathway from phosphatidylethanolamine (PE) catalyzed by PE-N-methyltransferase (PEMT), than on the cytidine diphosphate (CDP)-choline (Kennedy) pathway from choline catalyzed by cytidine triphosphate (CTP)-phosphocholine cytidyltransferase (CT) as the rate-limiting step in females.⁴¹ In fact, PEMT-2, is reportedly up-regulated as a compensatory pathway in male but not female livers with choline-deficiency.^{42, 43} In this context, the recent reports of inverse and positive correlations for PEMT-2 and CT, respectively, with hepatocyte proliferation during regeneration and in tumors in rats,^{44, 45} might be of interest. Further, estradiol has been reported to inhibit fibrosis induced by dimethylnitrosamine and pig serum, and the activation of cultured hepatic stellate cells in rats.⁴⁶ Moreover, androgen dependency of hepatocarcinogenesis has recently been postulated. Cytosolic androgen receptors are increased in human adenomatous nodules and HCCs⁴⁷ and the development of preneoplastic liver foci can be inhibited by either castration or estradiol treatment in male rats, while being enhanced in females by testosterone.⁴⁸ Thus, sexual dimorphism in PC metabolism, fibrosis and hepatocarcinogenesis might be involved in the present female resistance to the CDAA diet.

In conclusion, the present results provide the basis for an experimental model of hepatocarcinogenesis caused by endogenous factors in mice. Future studies using various gene-modified C57BL/6J mice should provide profound insights into the mechanisms underlying oxidative DNA damage, fibrosis and altered PC metabolism caused by a CDAA diet.

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