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High Incidence of Hepatocellular Carcinomas Induced by a Choline Deficient L-Amino Acid Defined Diet in Rats[1]

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ABSTRACT

The carcinogenicities of a choline deficient L-amino acid defined (CDAA) diet and a semipurified choline deficient diet were comparatively examined. A total of 60 male Fischer 344 rats, 6 weeks old, were divided into 5 experimental groups each consisting of 12 rats. Group 1 received the CDAA diet chronically to the end of the 52-week experiment while Group 2 was given the same diet for the first 24 weeks and then a basal diet for the following 28 weeks. Groups 3, 4, and 5 received a choline supplemented L-amino acid defined diet, the semipurified choline deficient diet, and a semipurified choline supplemented diet, respectively, throughout the experimental period. All surviving rats were subjected to complete macroscopic examination at Week 52. Histopathologically diagnosed hepatocellular carcinomas were induced in Group 1 at an incidence of 100%; multiple metastatic nodules were seen in the lungs of one of the animals. Hepatocellular carcinomas were also induced in Group 4 rats at a significantly lower incidence of 20%. No hepatocellular carcinomas were observed in rats in Groups 2, 3, and 4. The results indicate that the CDAA diet exerts more potent carcinogenicity for the livers of rats than does the semipurified choline deficient diet. However, limited exposure for 24 weeks may have not been sufficient for hepatocellular carcinoma induction by the CDAA diet at Week 52 although a high incidence of hyperplastic nodules and slight cirrhosis were evidence of persistent lesions.

INTRODUCTION

Hepatocarcinogenesis caused in rodents by prolonged dietary choline deficiency is unique since HCC[3] are induced without additional exposure of animals to chemical carcinogens either intentionally or adventitiously (1–4). The mechanisms underlying dietary choline deficiency associated neoplasia remain obscure although liver cell necrosis followed by regenerative cell proliferation (4–6), hypomethylation of nucleic acids (7–10), oncogene modification (11), and oxidative stress (3, 12–20) have all been suggested to play important roles.

Recently, we studied the comparative abilities of CDAA and CD diets to induce putative preneoplastic γ-glutamyltransferase positive liver lesions, altered hepatocyte foci and hyperplastic nodules, and oxidative DNA damage (20). It was found that the number and average size of induced preneoplastic lesions as well as the degree of oxidative DNA damage were significantly greater in the livers of rats receiving the CDAA diet than in the CD diet case (20). Those results suggested not only a possible contribution of oxidative stress to the hepatocarcinogenic mechanisms of dietary choline deficiency but also a stronger ability of the CDAA diet to induce preneoplastic liver lesions in rats than the CD diet. To confirm and extend these findings, we conducted the present longer-term experiment.

MATERIALS AND METHODS

Animals. A total of 60 male Fischer 344 rats, 5 weeks old, were obtained from Japan SLC Inc., Hamamatsu, Shizuoka, Japan, and housed, 3 to a stainless steel wired cage, in an air-conditioned room maintained at 25°C with a 12-h dark/light cycle. Access to food and tap water was *ad libitum* throughout the experiment. After a 1-week acclimation period on a basal diet in pellet form (Oriental MF Diet; Oriental Yeast Company, Limited, Itabashi, Tokyo, Japan), the animals were divided into experimental groups.

Diets. The CDAA, CSAA, CD, and CS diets were all in pellet form and obtained from Dyets Inc., Bethlehem, PA (Products 518753, 518754, 118753, and 118754, respectively). The detailed compositions of these diets are shown in Table 1. All diets contained 50 g/kg of corn oil and 100 g/kg of Primex as lipid sources. The amino acid composition of the CDAA or CSAA diet was made up only of pure L-amino acids with the exception of glycine which is not optically active. The average calorific contents of the CDAA, CSAA, CD, and CS diets were 4.32, 4.27, 4.43, and 4.37 kcal/g, respectively. The CDAA and CD diets both contained 6.5 mg/kg of choline and 1.75 g/kg of methionine. The CSAA and CS diets supplementarily included 14.48 g/kg of choline bitartrate. All diets were stored at 4°C immediately after arrival, and each batch was consumed at least within 1 month.

Experimental Protocol. The total experimental period was 52 weeks. The 5 experimental groups consisted of 12 rats each. Group 1 received the CDAA diet continuously to the end of the experiment. Group 2 received the CDAA diet during the first 24 weeks and then the basal diet for the following 28 weeks. Groups 3, 4, and 5 received the CSAA, CD, and CS diets, respectively, throughout the experimental period. All rats were weighed weekly during the first 8 weeks and biweekly thereafter. Diets were replaced every Monday and Friday. At the end of the experiment, all rats were sacrificed under light ether anesthesia and grossly examined. The livers were then immediately excised, weighed, fixed in an ice-cold mixture of dehydrated ethyl alcohol and glacial acetic acid at a ratio of 19:1 for 3 h followed by an overnight incubation in 99.5% ethyl alcohol at 4°C, and embedded in paraffin. Sections were processed routinely for hematoxylin and eosin staining and examined histopathologically. If the presence of extrahepatic metastases was suspected from macroscopic findings, the suspected organ(s) was (were) also excised, treated in a manner similar to that for the livers, and histopathologically examined.

Statistical Evaluation. The statistical significance of intergroup differences in quantitative data concerning body and liver weights and the incidences of liver lesions was determined with the aid of Student's *t* test and the χ^2 test, respectively.

RESULTS

In Groups 1, 2, 4, and 5, there were 2, 1, 2, and 1 rats, respectively, that died within the first 26 weeks; all deaths were caused by pneumonia. No spontaneous deaths occurred because of the presence of tumors or other specific pathological diseases. The effective numbers of rats in Groups 1, 2, 3, 4, and 5 were, therefore, 10, 11, 12, 10, and 11, respectively (Table 2). There were no intergroup differences in terms of food intake throughout the experimental period (data not shown). The data

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[3] The abbreviations used are: HCC, hepatocellular carcinoma(s); CDAA diet, choline deficient L-amino acid defined diet; CD diet, semipurified choline deficient diet; CSAA diet, choline supplemented L-amino acid defined diet; CS diet, semipurified choline supplemented diet; 80HdGuo, 8-hydroxydeoxyguanosine.

Table 1 *Diet Compositions*

Ingredient (g/kg diet)[b]	Diet[a]			
	CDAA[c]	CSAA	CD[c]	CS
L-Arginine	12.7	12.7		
L-Histidine	3.4	3.4		
L-Lysine-HCl	9.1	9.1		
L-Tyrosine	5.7	5.7		
L-Tryptophan	1.8	1.8		
L-Phenylalanine	7.3	7.3		
L-Methionine	1.7	1.7		
L-Cystine	3.7	3.7	2.0	2.0
L-Threonine	4.6	4.6		
L-Leucine	10.5	10.5		
L-Isoleucine	6.1	6.1		
L-Valine	6.3	6.3		
Glycine	6.2	6.2		
L-Proline	7.6	7.6		
L-Glutamic acid	28.9	28.9		
L-Alanine	5.1	5.1		
L-Aspartic acid	15.8	15.8		
L-Serine	7.2	7.2		
Alcohol-extracted peanut meal			90	90
Soy protein isolate			80	80
Vitamin free casein			10	10
Corn starch	100	100	100	100
Dextrin	100	100	100	100
Cellulose	50	50	10	10
Sucrose	407.00	392.52	413.00	398.52
Sodium bicarbonate	4.3	4.3		
Corn oil	50	50	50	50
Primex	100	100	100	100
AIN-76 salt mix	35	35	35	35
AIN-76A vitamin mix	10	10	10	10
Choline bitartrate		14.48		14.48

[a] Average calorific contents of the CDAA, CSAA, CD, and CS diets were 4.32, 4.27, 4.43, and 4.37 kcal/g diet, respectively.

[b] All of the amino acids were L forms except for glycine which is not optically active. Total amino acid (protein) contents in the CDAA and CSAA diets were the same as those in the CD and CS diets. L-Lysine in the latter two was substituted by L-lysine-HCl in an equimolar isonitrogenous manner in the L-amino acid defined diets. Sodium bicarbonate was included to neutralize this hydrochloride.

[c] Contained 6–7 mg/kg of choline and 1.7–1.8 g/kg of methionine.

for initial body weights, final body weights, and liver weights relative to 100 g body weight of each group are summarized in Table 2. The final body weights for both Groups 3 and 4 were significantly higher than those of Groups 1 and 2 and lower than that of Group 5. Relative liver weights for Groups 2, 3, and 4 were all less than that of Group 1. The weights of Groups 3 and 4 were significantly lower and higher, respectively, than that of Group 2. The Group 4 value was significantly higher than that of Group 5.

Macroscopically, the livers of all Group 1 rats were yellowish and appeared cirrhotic in association with turbid and dark yellowish-white nodules, occasionally accompanied by central ulceration. The typical macroscopic appearance of a Group 1 liver is shown in Fig. 1. The livers of Group 2 animals also looked cirrhotic but lacked turbid nodules, and they were brownish-purple. The livers of Group 3 rats were brownish-purple and smooth, evidencing no pathological change. The livers of Group 4 rats resembled those in Group 1 rats, but turbid nodules were seen in only 2 animals. Group 5 rats demonstrated normal appearing livers like those in Group 3.

Table 2 shows the incidences of HCC, hyperplastic nodules, and cirrhosis histopathologically diagnosed at the end of the 52-week experiment in the livers of rats in each group. One hundred % of Group 1 rats developed HCC, their typical histopathological appearance is illustrated in Fig. 2. Relatively well differentiated cancer cells displayed chiefly trabecular and occasionally pseudoglandular patterns. Portal invasion of cancer cells was sometimes seen while invasion of bile ducts was less frequent. In one Group 1 rat, multiple metastatic nodules of HCC were detected in the lungs (Fig. 3). Hyperplastic nodules and fatty cirrhosis were also features of all livers of Group 1 rats. In Group 2, no HCC were found, but hyperplastic nodules and slight fatty cirrhosis were seen at respective incidences of 73 and 82%. No histopathological changes were detected in the livers of any animals of Group 3 and 5 rats. Although HCC were also induced in Group 4, the incidence of 20% was significantly lower than that obtained in Group 1 after 52 weeks. Hyperplastic nodules and fatty cirrhosis were both found in all Group 4 rats.

DISCUSSION

The results of the present study indicated that: (*a*) a choline deficient L-amino acid defined diet exerts more potent carcinogenicity on the livers of male Fischer 344 rats than a semipurified choline deficient diet containing the same amounts of choline and methionine; and (*b*) a 24 week CDAA diet regimen may be insufficient for HCC induction.

The former indication confirmed and extended the results of our previous comparative study concerning the abilities of the CDAA and CD diets to induce preneoplastic liver lesions (20). The incidence of HCC development in male Fischer 344 rats fed the CD diet has been reported in the literature to be 51% after 13–24 months (2), 22% after 36–48 weeks (21), and 26% after 16 months (4). Thus, the present incidence of HCC obtained in the livers of rats fed the CD diet, 20%, for 52 weeks is well in accordance with those earlier reports. Although the reason that the CDAA diet expresses so much greater carcinogenicity than does the CD diet has not yet been completely elucidated, it cannot be attributed simply to the contents of choline and methionine in diets since the CDAA and CD diets contain the same amounts of those nutritional elements. It is possible that the lack of oligopeptides in the CDAA case causes less transintestinal absorption of methyl donor amino acids and

Table 2 *Data for effective numbers, initial and final body weights, relative liver weights, and incidences of liver lesions*

Group	Diet(s)	Effective no. of rats	Body wt (g)		Relative liver wt (g/100 g body wt)	Incidence of liver lesions		
			Initial	Final		HCC	Hyperplastic nodule	Cirrhosis
1	CDAA	10	166 ± 3[a]	413 ± 26[a]	4.96 ± 0.38[a]	10/10 (100)[b]	10/10 (100)[b]	10/10 (100)[b]
2	CDAA for 24 weeks and then basal for 28 weeks	11	167 ± 5	416 ± 15	3.25 ± 0.24[c]	0/11 (0)[c]	8/11 (73)	9/11 (82)
3	CSAA	12	169 ± 4	485 ± 25[c–e]	2.80 ± 0.34[c,d]	0/12 (0)[c]	0/12 (0)[c,d]	0/12 (0)[c,d]
4	CD	10	168 ± 2	481 ± 26[c–e]	4.03 ± 0.81[c–e]	2/10 (20)[c]	10/10 (100)[e]	10/10 (100)[e]
5	CS	11	170 ± 6	538 ± 43	2.73 ± 0.14	0/11 (0)	0/11 (0)	0/11 (0)

[a] Mean ± SD.

[b] Numbers of rats with lesions/total effective numbers. Numbers in parentheses, percentage incidences.

[c] $P < 0.01$ *versus* Group 1.

[d] $P < 0.01$ *versus* Group 2.

[e] $P < 0.01$ *versus* Group 5.

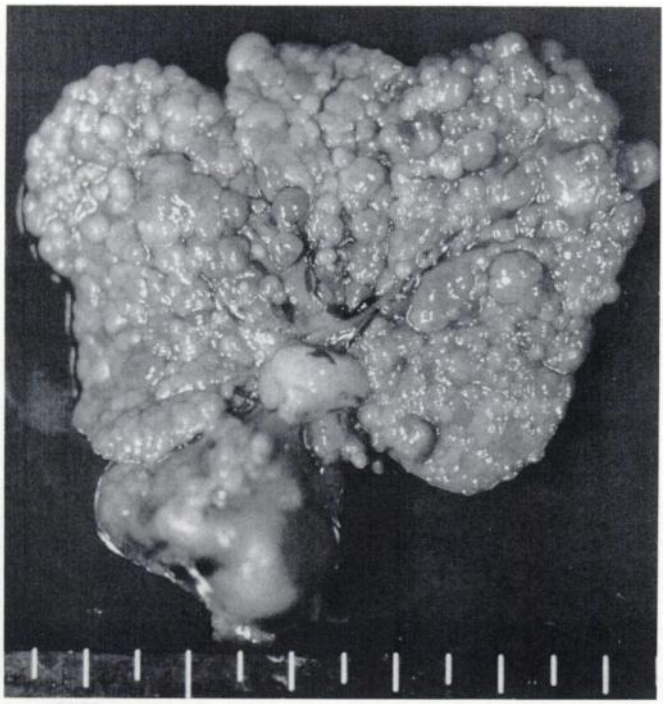

Fig. 1. Typical macroscopic appearance of the liver of a rat fed the CDAA diet continuously for 52 weeks (Group 1).

antioxidant minerals resulting in a more severe methyl deficient state with concomitant lowering of antioxidant defenses as discussed previously (20). Alternatively, the CDAA diet might be more necrogenic and, subsequently, capable of inducing more progressive regenerating cell proliferation than is the CD diet. A sequence of liver cell necrosis and regenerating cell proliferation has been thought to play a critical role in liver carcinogenesis caused by dietary choline deficiency (4–6); therefore, if the CDAA diet is indeed associated with more toxicity than is the CD diet, this difference could help explain the observed difference in carcinogenicity. In fact, cirrhosis associated with the development of hyperplastic nodules was already present in the livers of rats given the CDAA diet after only 12 weeks at which time lesions in the CD diet fed animals were limited to fibrosis along with altered hepatocyte foci (20). Another highly necrogenic diet also deficient in choline but with a considerably different composition from those of the CDAA and CD diets, namely, a methyl deficient amino acid defined diet, also demonstrated strong carcinogenicity. Sawada *et al.* (22) administered this diet to male Fischer 344 rats and gained 70% incidence of HCC development 24 weeks after the commencement of treatment while we could not detect any HCC development in rats fed the CDAA diet for 24 weeks.[4] The methyl deficient amino acid defined diet did not contain any choline and methionine (1). It is, however, again difficult to simply attribute the difference between the two amino acid defined diets in terms of their abilities for HCC development to that of the contents of choline and methionine between those two diets; *i.e.*, the methyl deficient amino acid defined diet has a considerably different amino acid composition, contains more dextrin and salt mixture, lacks corn starch and cellulose, and includes corn oil as an only lipid source when compared with the CDAA diet (1). These various nutritional differences could affect the tumorigenicities of the diets from a variety of directions. Furthermore, Mikol *et al.* (1) reported that the methyl deficient amino acid defined diet developed HCC in male Fischer 344 rats at an incidence of only 36% for 77 weeks, which indicates a relatively unstable carcinogenic potential for this diet.

The second finding from the present study suggests that a continuous stimulus of dietary choline deficiency may be required in order for preneoplastic lesions to grow into HCC. Diets deficient in choline are well known to demonstrate promoting potential (1, 3, 5, 21–23).

We reported earlier that both the CDAA and CD diets induce oxidative liver DNA damage detected as 80HdGuo as well as preneoplastic liver lesions, inasmuch as the abilities of the CDAA diet to induce 80HdGuo and such lesions are correlatively greater than those of the CD diet (20); that 80HdGuo in liver DNA becomes detectable 3 days after the commencement of the feeding of the CDAA diet and is accumulated at least up to Week 12 (24); and that the inductions of both 80HdGuo and preneoplastic lesions in the livers of rats fed the CDAA diet for 12 weeks are inhibited by dietary iron deficiency (24). This might indicate an essential role of 80HdGuo in hepatocarcinogenesis, but it must be kept in mind that this could simply be a reflection of stronger necrogenicity. Moreover, despite a recent accumulation of evidence for the relevance of

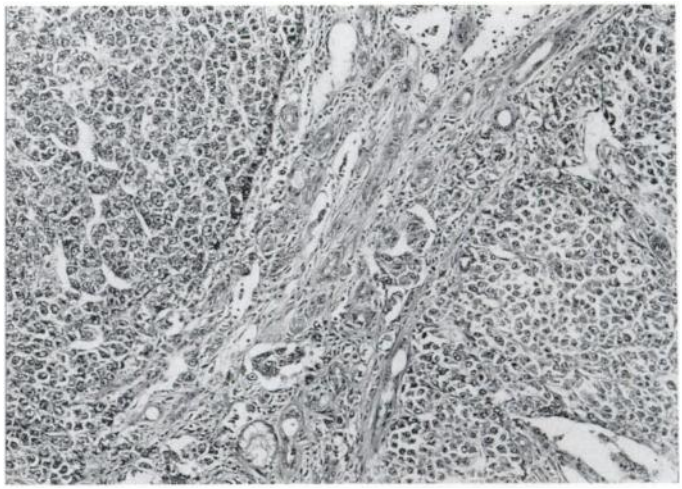

Fig. 2. Typical histopathological appearance of a HCC found in a Group 1 rat. H & E, × 10.

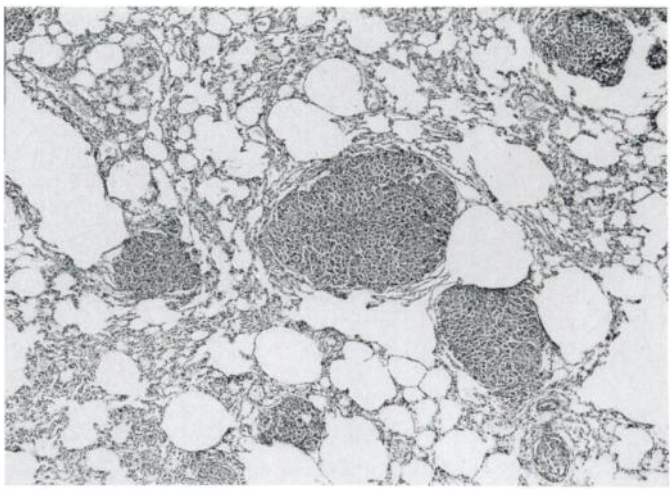

Fig. 3. Multiple metastatic lung nodules from a HCC found in a rat fed the CDAA diet continuously for 52 weeks (Group 1). H & E, × 5.

[4] Unpublished results.

80HdGuo to mutagenesis and/or carcinogenesis (25–29), the significance and exact role played by this "oxidative" DNA damage in the course of carcinogenesis is not yet fully understood. In this context, investigations focusing on the relationship between the CDAA diet associated oxidative DNA damage and cell necrosis both in normal appearing parenchyma and within preneoplastic lesions and/or HCC are necessary. Such studies are now under way in our laboratory.

Finally, it should be noted that the participation of oxidative stress in rat hepatocarcinogenesis by dietary choline deficiency has not yet generally accepted. This idea arose from reports showing the induction of peroxidation of membrane lipids in various subcellular fractions of hepatocytes in the course of liver carcinogenesis by dietary choline deficiency (3, 12–14). Banni *et al.* (30, 31), however, recently cast doubt on the induction of such hepatocellular membrane lipid peroxidation by the CD diet. Nevertheless, we demonstrated that the CDAA diet indeed induces the peroxidation of liver cell membrane lipids time dependently, and the peroxidation is inhibited by dietary iron deficiency (24). The results in terms of 80HdGuo reported by us (20, 24) and Hinrichsen *et al.* (19) considered together make it conceivable that oxidative stress might, at least in part, participate in the mechanisms underlying rat liver carcinogenesis by dietary choline deficiency.

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