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Prevention by Methionine of Enhancement of Hepatocarcinogenesis by Coadministration of a Choline-deficient L-Amino Acid-defined Diet and Ethionine in Rats

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The effects of methionine on hepatocarcinogenesis induced by coadministration of a choline-deficient L-amino acid-defined (CDAA) diet and ethionine were examined. F344 male rats were divided into 4 experimental groups. Groups 1 and 2 received the CDAA diet and a choline-supplemented L-amino acid-defined (CSAA) diet, respectively. Group 3 received the CDAA diet containing 0.05% ethionine, and group 4 the CDAA diet containing 0.05% ethionine and 0.47% methionine. Animals were killed after 12 weeks of treatment. Histologically, the CDAA diet induced intracellular fat accumulation and foci. In contrast, ethionine caused not only foci, but also hyperplastic nodules, cholangiofibrosis and the proliferation of oval cells without such fat accumulation. Methionine abolished the development of all of the liver lesions induced by coadministration of the CDAA diet and ethionine. To investigate the effects of methionine on induction of c-*myc* and c-Ha-*ras* expression, as well as generation of 8-hydroxyguanine (8-OHGua) and 2-thiobarbituric acid-reacting substances (TBARS), by coadministration of the CDAA diet and ethionine, subgroups of 3 to 5 animals were killed at 2, 4, 8 or 11 days after the beginning of the experiment. Coadministration of the CDAA diet and ethionine markedly enhanced the level of expression of c-*myc* and c-Ha-*ras*, 8-OHGua formation and TBARS generation as compared with the CDAA or CSAA diet within 11 days, and methionine blocked these actions. These results indicate that addition of methionine prevents the induction of c-*myc* and c-Ha-*ras* expression, 8-OHGua formation and TBARS generation, as well as hepatocellular lesions, by coadministration of the CDAA diet and ethionine in rats, and suggest a possible involvement of oxidative stress and gene expression in hepatocarcinogenesis by these agents.

Key words: Choline-deficient L-amino acid-defined diet — Ethionine — Methionine — Hepatocarcinogenesis — Rat

The fact that unequivocal liver tumors can be induced by prolonged feeding of rats with the CD diet[2] is well-known.[1–3] Possible mechanisms underlying liver carcinogenesis by the CD diet have been proposed to be as follows: liver cell necrosis associated with subsequent regeneration[4, 5]; induction of oxidative DNA damage[6, 7] and lipid peroxidation[8, 9]; and generation of gene alterations.[10, 11] Recently, we have developed a new model for rat hepatocarcinogenesis due to dietary choline deficiency with the CDAA diet, in which the amino acid composition of the CD diet is defined by using pure L-amino acids, and observed a stronger carcinogenic action than with the CD diet in rats.[12] Although oxidative stress has been considered as an important candidate, the mechanisms underlying hepatocarcinogenesis by the CDAA diet in rats have not been fully clarified as yet. Coadministration of ethionine, which is a potent liver carcinogen in rats,[13] with the CD diet has been shown to enhance the development of hepatocellular lesions as compared to the outcome with either agent alone.[14, 15] Since ethionine interferes with methionine metabolism, resulting in an imbalance of S-adenosylmethionine and S-adenosylethionine,[13] the level of methyl donor, which exerts numerous effects on cell functions and metabolism, might be related to carcinogenic potency in the rat liver.

In the present study, to investigate this question, the effects of methionine on hepatocarcinogenesis induced by coadministration of the CDAA diet and ethionine in rats were studied in terms of changes in expression of c-*myc* and c-Ha-*ras*, 8-OHGua formation and TBARS generation as parameters, in addition to focal lesion development.

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[2] Abbreviations: CD diet, a semipurified choline-deficient diet; CDAA diet, a choline-deficient L-amino acid-defined diet; CSAA diet, a choline-supplemented L-amino acid-defined diet; 8-OHGua, 8-hydroxyguanine; 8-OHGuo, 8-hydroxydeoxyguanosine; dGuo, deoxyguanosine; TBARS, 2-thiobarbituric acid-reacting substances; MDA, malondialdehyde; GGT, γ-glutamyltransferase.

MATERIALS AND METHODS

Animals A total of 234 male Fischer 344 rats (Shizuoka Laboratory Animal Center, Shizuoka), 6 weeks old, were used. The animals were housed five per plastic cage in an air-conditioned room at 24°C and 60% humidity with a daily 12-h alternating cycle of dark and light, and given food and water *ad libitum*.

Diet and chemicals CDAA and CSAA diets, with compositions as described previously,[12] were purchased from Dyets Inc., Bethlehem, PA (Product numbers 518753 and 518754, respectively). They were stored at 4°C immediately after arrival, and the experiment was completed with a single batch of each diet. Special grade DL-ethionine and L-methionine were obtained from Nacalai Chemical Co. Ltd., Kyoto.

Treatments The protocol used in this study is shown in Fig. 1. Animals from groups 1 and 2 received the CDAA diet and the CSAA diet, respectively. Group 3 received the CDAA diet containing 0.05% ethionine, while group 4 was given the CDAA diet containing 0.05% ethionine and 0.47% methionine. These treatments were continued throughout the experimental period. Body weights and food intakes of animals from each group were measured weekly. Twelve weeks after the commencement of the experiment, all animals were killed under ether anesthesia and livers were immediately excised. For assessment of the effects of methionine on expression of c-*myc* and c-Ha-*ras*, 8-OHGua formation and TBARS generation in the liver of rats fed the CDAA diet containing ethionine, subgroups of 3 to 5 animals were killed at 2, 4, 8 or 11 days after the beginning of the experiment. Livers were removed immediately, frozen in liquid nitrogen and stored at −80°C until used.

Histological and histochemical studies Liver slices taken from different lobes were fixed in 95% ethanol containing 1% acetic acid for 2 h at 4°C and then overnight in ethanol at 4°C. Liver tissues were routinely processed and paraffin-embedded sections were stained with hematoxylin and eosin, and were histopathologically diagnosed according to the criteria of Squire and Levitt.[16] GGT was stained using the method of Rutenberg *et al.*[17] The numbers and sizes of GGT-positive foci were analyzed with an image analyzer model HTB-c955 (Hamamatsu Television Co. Ltd., Shizuoka) connected to a Desktop Computer System 45 (Hewlett-Packard Co., USA). The numbers of GGT-positive foci per cm^3 were calculated using the formula of Campbell *et al.*[18] Even the smallest positively stained lesions had an area of 0.08 mm^2. Data were statistically analyzed by using Student's *t* test.

Determination of 8-OHGua formation in DNA DNA was isolated from pooled liver samples (approximately 2 g wet weight) of 3 to 5 rats, and digested into deoxynucleosides by combined treatment with nuclease P1 (Yamasa Shoyu Co., Ltd., Chiba) plus alkaline phosphatase (Sigma, St. Louis, MO) by the method of Takagi *et al.*[19] The level of 8-OHGuo in each resultant preparation was determined by high-performance liquid chromatography with electrochemical detection by an adaptation of the methods of Floyd[20] and Kasai *et al.*[21] An authentic sample of 8-OHGuo was generously provided by Dr. Hiroshi Kasai (Department of Environmental Oncology, Institute of Industrial Ecological Sciences, University of Occupation and Environmental Health, Fukuoka), and dGuo was obtained from Sigma for control purposes. Levels of 8-OHGua formation in DNA were expressed as the numbers of 8-OHGuo formed per 10^5 total dGuo nucleosides.

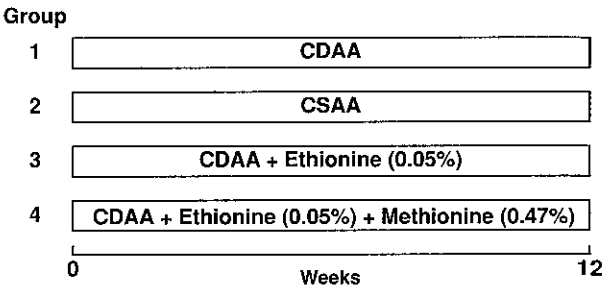

Fig. 1. Experimental protocol.

Determination of TBARS generation Levels of TBARS generation in total pooled liver homogenates from 3 to 5 rats were assessed by an adaptation of the method of Yagi[22] as described previously.[23] The fluorescence intensities of the resultant samples at 553 nm (emission) and 515 nm (excitation) were collated against those of standard amounts of MDA (Aldrich Chemical Co., Inc., Milwaukee, WI) and standardized for protein values measured using the BCA Protein Assay with bicinchoninic acid (Pierce Chemical Co., Rockford, IL).

Northern blot analysis Ten μg of total cytoplasmic RNA was isolated from pooled livers from 3 to 5 rats using the lithium chloride-urea method[24] and subjected to electrophoresis on 1% agarose/formaldehyde gels. The gels were capillary-blotted in 20× standard saline citrate onto Biodyne A nylon membranes (Poll Biosupport Co., East Hills, NY), and baked at 80°C for 1 h.

Preparation of cDNA probes The human c-*myc* exon3 probe was a 1.5 kb *Eco*R I-*Cla* I fragment of plasmid pMCE2.[25] The mouse c-Ha-*ras* probe was a 0.4 kb *Eco*R I fragment of Ha-MusV inserted into plasmid pBS-9.[26] These plasmids were grown in *Escherichia coli* host HB101. The procedures used for cDNA plasmid-insert preparation were as described by Sambrook *et al.*[27]

RESULTS

Number of animals, body weights, liver weights and food intake Animal numbers, body weights, liver weights and food intake data are shown in Table I. Ethionine-treated animals exhibited significant inhibition of body and liver weights as shown in group 3 compared with groups 1 and 2, but this was no longer the case with addition of methionine. The addition of ethionine and/or methionine significantly decreased the relative liver weight per body weight as compared with the CDAA diet alone. The average intake of food in group 4 was lower than in the other groups throughout the experiment.

Effects of methionine on induction of GGT-positive foci and liver lesions by coadministration of the CDAA diet and ethionine Numbers and areas of GGT-positive liver lesions are shown in Table II. Ethionine increased the % area occupied by foci and their size, although the numbers per cm^2 and cm^3 of GGT-positive lesions were low as compared with the CDAA diet alone case. With addition of methionine, no induction of GGT-positive lesions could be observed. The CSAA diet did not induce any GGT-positive liver lesions.

Histopathological findings are summarized in Table III. The CDAA diet induced fat accumulation and foci in all rats. Ethionine induced not only foci but also hyperplastic nodules with cholangiofibrosis, although no fat accumulation was evident. Proliferation of oval cells was found with the CDAA diet containing ethionine, but not with the CDAA diet alone. The addition of methionine resulted in only slight fat accumulation of hepatocytes without any other changes. The CSAA diet caused no liver lesions.

8-OHGua formation and TBARS generation Typical data for 8-OHGua formation in DNA at 4 and 11 days after the beginning of the study and TBARS generation in total liver homogenates at 4 and 11 days are shown in Table IV. The levels of 8-OHGua in liver DNA and TBARS generation for rats fed the CDAA diet containing ethionine were greater than in those given the CDAA

Table I. Details of Body and Liver Weights, and Food Intake

Experimental group	No. of rats	Body weight (g)[a]	Liver weight (g)[a]		Average food intake (g/rat/day)[a]
		Final	(g)	Ratio to body weight ($\times 10^2$)	
1. CDAA	12	234.4±22.8	10.16±2.00	4.33±0.65	22.9±5.1
2. CSAA	12	279.9±11.6[c, e]	6.60±0.32[c, e]	2.36±0.10[c, e]	23.0±5.4
3. CDAA+ethionine	11	102.7±8.3[c]	3.96±0.42[c]	3.86±0.40[b]	18.9±5.8
4. CDAA+ethionine +methionine	13	286.6±14.2[c, e]	7.47±0.59[c, e]	2.61±0.09[c, e]	14.6±3.7[c, d]

a) Data shown are mean±SD.
b) Significantly different from group 1, $P<0.05$.
c) Significantly different from group 1, $P<0.001$.
d) Significantly different from group 3, $P<0.05$.
e) Significantly different from group 3, $P<0.001$.

Table II. Effects of Coadministration of the CDAA Diet and Ethionine on the Development of GGT-positive Liver Lesions in Rats

Experimental group	GGT-positive lesions[a]			
	Number /cm^2	% Area occupied by foci	Number /cm^3	Size (mm^3)
1. CDAA	1.84±1.09	1.58±1.33	32.1±19.9	0.92±2.02
2. CSAA	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]
3. CDAA +ethionine	0.93±0.98	4.22±6.55	10.8±13.6[b]	17.3±55.3
4. CDAA +ethionine +methionine	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]

a) Data shown are mean±SD.
b) Significantly different from group 1, $P<0.01$
c) Significantly different from group 1, $P<0.001$.

Table III. Incidence of Histologic Liver Lesions Induced by the CDAA Diet and Ethionine in Rats[a)]

Experimental group	Fatty change		Focus	Hyperplastic nodule	Cholangio-fibrosis
	Focal	Diffuse			
1. CDAA	0 (0)	12 (100)	12 (100)	0 (0)	0 (0)
2. CSAA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3. CDAA+ethionine	0 (0)	0 (0)	10 (90.0)	3 (27.3)	11 (100)
4. CDAA+ethionine +methionine	13 (100)	0 (0)	0 (0)	0 (0)	0 (0)

a) The numbers in parenthesis are the corresponding percentages.

Table IV. The Formation of 8-OHGua in DNA and TBARS Generation by Coadministration of the CDAA Diet and Ethionine in Rat Liver

Experimental group	8-OHGua formation (8-OHdGuo/10^5 dGuo)		TBARS generation (pmol MDA equivalent/mg protein)	
	4[a)]	11	4	11
1. CDAA	3.18	3.98	9	11
2. CSAA	1.52	1.51	4	3
3. CDAA+ethionine	6.73	8.81	20	23
4. CDAA+ethionine+methionine	3.75	3.74	5	5

a) Treatment period (days).

diet alone. However, methionine reduced both 8-OHGua formation and TBARS generation to the CSAA diet levels.

Expression of c-*myc* and c-Ha-*ras* induced by coadministration of the CDAA diet and ethionine Results for expression of c-*myc* and c-Ha-*ras* are shown in Fig. 2, both being elevated by the CDAA diet alone as compared to the CSAA diet case 2 days after the beginning of the experiment, and these expressions were maintained to 11 days. Moreover, ethionine enhanced the expression of both genes during the same periods. Methionine, in contrast, caused a decrease in the expression levels of both genes to these found with the CSAA diet. The presence of equivalent amounts of RNA in each lane was confirmed by ethidium bromide staining (data not shown).

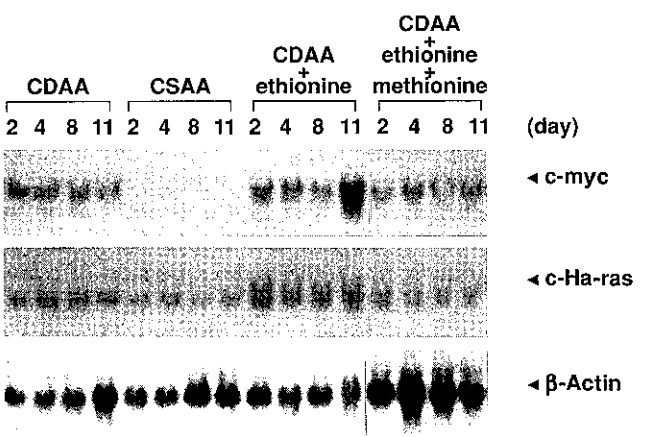

Fig. 2. The expressions of c-*myc* and c-Ha-*ras* in liver of rats coadministered with the CDAA diet and ethionine.

DISCUSSION

The present study showed that methionine can prevent hepatocarcinogenesis due to coadministration of the CDAA diet and ethionine in rats. Ethionine is known to be a potent liver carcinogen in rats, causing unequivocal liver cancers at a 0.25% dose mixed into basal diet.[13)] Moreover, coadministration of lower doses of ethionine and the CD diet has a stronger carcinogenic potency in rat liver.[14, 15)] It has been reported that the addition of methionine exerts a prevention effect on hepatocarcinogenesis induced by ethionine in rats.[28)] Therefore, the available evidence, including the present results, supports the conclusion that the carcinogenicity might be dependent on the degree of methyl-donor deficiency in rat liver.

Recently, we have reported accumulation of oxidative stress, assessed in terms of 8-OHGua formation and TBARS generation, during rat hepatocarcinogenesis induced by feeding the CDAA diet.[29)] Furthermore, antioxidants, such as 2-O-octadecylascorbic acid (CV3611) and DPPD, were found to inhibit the development of liver lesions in this model.[29, 30)] Thus, the possible involvement of oxidative stress has been suggested. In the pres-

ent study, coadministration of the CDAA diet and ethionine resulted in elevated 8-OHGua formation and TBARS generation as compared with the CDAA diet alone, and the addition of methionine to this regimen abolished their induction, along with development of focal lesions. Therefore, this provides direct evidence for a role of oxidative stress in hepatocarcinogenesis by these agents.

Such oxidative stress is known to induce alterations in expression of c-*fos*, c-*myc* and c-Ha-*ras* genes in rodents.[31, 32] Furthermore, 8-OHGua, which is known to be a major oxidative adduct,[19, 20, 33, 34] can cause base transversions of specific types.[35, 36] Recently, amplification of the c-*myc* gene and *p53* gene mutation in hepatocellular carcinomas induced by the CD diet in rats have been reported.[10, 11] Therefore, the possibility that alterations in several control genes might occur due to oxidative stress associated with the CD or CDAA diets deserves consideration. In the present study, coadministration of the CDAA diet and ethionine resulted in elevated induction of not only the markers of oxidative stress, but also the expression of c-*myc* and c-Ha-*ras*. Furthermore, methionine supplementation inhibited this in both cases. Oxidative stress causation of lethal injury to rat hepatocytes, followed by regenerative cell proliferation, has been reported.[4, 5] This might be related to the recent demonstration that short-term feeding of a methyl-deficient diet can induce high expression of several genes, including c-*fos*, c-*myc* and c-Ha-*ras*, and hypomethylation of DNA and tRNA in rat liver. Moreover, although these parameters returned to control levels after ending the methyl-deficient diet feeding, hypomethylation of specific sites in c-*fos*, c-*myc* and c-Ha-*ras* persisted.[37, 38] Therefore, in our findings, the high expression of c-*myc* and c-Ha-*ras* in an early stage of hepatocarcinogenesis might be simply due to regenerative changes. Hypomethylation of control genes might be necessary for induction of early focal lesions by methyl-deficiency, rather than elevated expression of the same genes, although CD diet-induced c-*myc* amplification has been detected in hepatocellular carcinomas in rats.[10] However, in the present study, we could not detect any hypomethylation of c-*myc* and c-Ha-*ras* in animals exposed to the CDAA diet containing ethionine for 11 days (data not shown).

Recently, activation of protein kinase C by fatty changes associated with accumulation of 1,2-sn-diradylglycerol in livers of CD diet-fed rats has been described.[39] In the present study, although biochemical analysis has not been performed as yet, histologically, no induction of fatty change was evident in animals receiving the CDAA diet containing ethionine. Therefore, the mechanisms underlying rat hepatocarcinogenesis might be different between the CDAA diet cases with and without ethionine.

Previous reports have documented that the CD diet exerts promoting effects on rat hepatocarcinogenesis initiated by chemical carcinogens, including ethionine, diethylnitrosamine and 2-acetylaminofluorene.[14, 15, 40, 41] Therefore, to clarify what role ethionine might play in the present combined treatment model, we should investigate whether oxidative stress can also be induced by administration of ethionine alone in rat livers. Furthermore, to obtain more information on the mechanism of enhanced carcinogenesis with coadministration of the CDAA diet and ethionine, examination of the gene alterations in induced hepatocellular lesions is warranted.

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