Alteration of Hepatic DNA Synthesis in Rats Treated with *N*-Nitrosodimethylamine during Early Stages of Copper Deficiency

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The main aim of this research was to clarify that nutritional dietary copper may participate in the protective action against hepatocarcinogenesis in rats by *N*-nitrosodimethylamine (NDMA). The copper concentrations in serum and liver from 2 to 8 d after rats were first fed a copper deficient diet (copper, 0.6 ppm) decreased significantly compared to those of pair-fed rats (copper in a control diet, 7 ppm). The subcellular distribution of copper in the liver at 5 d after feeding of a copper deficient diet began was measured and the copper concentrations in soluble and nuclear fractions decreased at a similar rate in copper deficient rats treated with or without NDMA, compared to those of pair-fed rats. The incorporation of [^{3}H]thymidine into rat liver DNA at 48 h after treatment with NDMA markedly increased under the experimental conditions used. By giving rats a copper deficient diet for a few days the increased incorporation of [^{3}H]thymidine into liver DNA of rats treated with NDMA was enhanced compared to that of pair-fed rats treated with NDMA. The activity of thymidine kinase in liver of copper deficient rats treated with NDMA was also found to increase significantly compared to that of pair-fed rats treated with NDMA.

Keywords *N*-nitrosodimethylamine; rat liver DNA synthesis; copper deficiency

Introduction

Excessive administration of copper reportedly has a suppressive effect against hepatocarcinogenesis in rats by chemical carcinogens such as aminoazo dyes,[1] ethionine,[2] and *N*-nitrosodimethylamine (NDMA).[3] The administration of NDMA which is an acute hepatotoxin and a potent carcinogen in many species[4–6] markedly increases the thymidine incorporation into liver DNA of rats.[7,8] We have reported[3,9,10] that the administration of copper to rats significantly suppresses NDMA-stimulated DNA synthesis in rat liver and hepatocarcinogenesis by NDMA. In contrast, it is suggested that the DNA synthesis in liver of rats stimulated by NDMA may be enhanced more in copper deficient rats than in pair-fed rats. The effect of copper deficiency on thymidine incorporation into liver DNA of rats treated with NDMA remains to be examined.

As an experiment to clarify whether nutritional dietary copper is participating in the protective action against hepatocarcinogenesis in rats by NDMA, we examined the alteration of hepatic DNA synthesis in animals stimulated by NDMA during early stages of copper deficiency.

Materials and Methods

Schedules of Experiment and Preparation of Diets Male Wistar rats were obtained from Matsumoto Labo-Animals Laboratory and maintained on the commercial diet CE-2 (Clea Japan Inc.). Rats weighing 95±5 g at the start of administration of an experimental diet were used. Each rat in a stainless steel cage was fed on a copper deficient diet and given distilled water freely. The pair-fed rats were used to assure against changes in thymidine kinase activity and incorporation of thymidine into DNA in the liver by different dietary intake, and given the same amount of normal diet as the average amount of an ingested copper deficient diet per rat. NDMA (Wako Pure Chemical Co.) was dissolved in 0.9% NaCl solution. Rats were given a single intraperitoneal injection of NDMA (30 mg/kg of body weight) at 3 or 7 d after they had first been given a copper deficient diet. They were sacrificed at 48 h after administration of NDMA, that is, at 5 or 9 d after starting the experimental diet. At 2, 4, 6 and 8 d after starting the diet the animals were exsanguinated by cardiac puncture and then the livers were perfused with 1.15% KCl solution and immediately removed. For each determination of copper concentration in serum and liver 6—8 rats were used. All rats used for measurement of the thymidine incorporation into DNA and the activity of thymidine kinase were starved for 24 h before sacrifice from 9:00 to 10:00 a.m. The copper deficient diet used in the experiment was prepared in our laboratory and had the following composition,[11,12] in percent: spray-dried egg white, 26.5; anhydrous dextrose, 56.4; corn oil, 10.0; mineral mix, 5.4; vitamin mix, 1.0; DL-methionine, 0.5; choline chloride, 0.2. Composition of basal mineral mix (grams) was as follows: $CaHPO_4 \cdot 2H_2O$, 639; NaCl, 108; K_2SO_4, 38.6; K_2CO_3, 631.4; $K_3C_6H_5O_7 \cdot H_2O$, 88.3; $MnCl_2 \cdot 4H_2O$, 3.8; $FeC_6H_5O_7 \cdot 5H_2O$, 5.1; $MgSO_4$, 52.7; $ZnSO_4 \cdot 7H_2O$, 17.2; $CrK(SO_4)_2 \cdot 12H_2O$, 0.41; KIO_3, 0.0074; Na_2SeO_4, 0.0057. Copper-supplemented mineral mix included the addition of 0.3 g of $CuCl_2 \cdot 2H_2O$. Composition of vitamins (miligrams) was as follows: thiamin·HCl, 200; riboflavin, 200; pyridoxine·HCl, 200; Ca-pantothenate, 400; *d*-biotin, 40; niacin, 500; folic acid, 40; cyanocobalamin (0.1% in mannitol), 500; menadion, 10; vitamin A palmitate (1000000 IU/g), 250; calciferol (40000000 IU/g), 1; DL-α-tocopherol, 649.3. Vitamins were added with glucose to a total of 200 g and the mixture was used as a vitamin mix. Copper concentration in the copper deficient diet was 0.6 mg of copper/kg of diet. Pair-fed rats were given the diet containing 7 mg of copper/kg of diet.

Determination of [^{3}H]Thymidine Incorporation into Liver DNA Rats were given 370 KBq of [^{3}H]thymidine (72×10^3 MBq/mmol, New England Nuclear, Boston, Mass)/100 g of body weight intraperitoneally 1 h before sacrifice. The liver was perfused with 1.15% KCl to remove blood, and moisture was wiped off. A slightly modified method of Schmidt–Thannhauser–Schneider was used.[9,13] Radioactivity of [^{3}H]thymidine in the DNA fraction was measured in triton–toluene scintillator by a liquid scintillation counter (Beckman LSC 5800). The content of DNA was measured according to Burton's method.[14]

Determination of Thymidine Kinase Activity in Liver The activity of thymidine kinase was determined according to the method of Bresnick and Karjala.[15] The liver homogenate was centrifuged at 105000*g* for 60 min and the supernatant was used as enzyme source. The reaction mixture included the enzyme solution: 2.94 μM [^{3}H]thymidine (74×10^3 MBq/mmol); ATP, 5 mM; $MgCl_2$, 2.5 mM; and 0.05 M Tris buffer, pH 8.0, in a total volume of 0.25 ml. Incubation was performed at 37 °C for 10 min. The reaction was then stopped by heating the mixture at 100 °C for 3 min. Aliquots of the reaction mixture were applied to discs of DEAE-cellulose (DE-81, Whatman), 2.5 cm in diameter, and the [^{3}H]thymidine was eluted from these discs with ammonium formate solution. The discs on which the [^{3}H]thymidinemonophosphate produced from [^{3}H]thymidine was retained were placed in methanol, dried at 80 °C, immersed in a triton–toluene scintillator, and counted in a liquid scintillation counter. The protein concentration of the enzyme solution was estimated by the method of Lowry *et al.*[16] using bovine serum albumin as the reference standard.

Determination of Metal Concentration in Serum and in Liver Subcellular Fractions The serum was prepared by centrifuging the blood at 3000*g* for 10 min. The livers were wiped off, minced and homogenized. The subcellular fractionation was performed by centrifugation of the liver

homogenate.[9,17] All materials including serum were decomposed by wet ashing using sulfuric and nitric acids.[9,10] The concentration of metals was determined by atomic absorption spectrophotometry[9,10] as follows. The sample solution, after addition of 10 ml of 25% dibasic ammonium citrate and 2 drops of 0.1% bromothymol blue, was adjusted to approximately pH 7.6 by adding ammonium water, and 5 ml of 10% diethyldithiocarbamate was added to the mixture. The mixture was shaken, then 10 ml of methylisobutylketone (MIBK) added, and the whole was vigorously shaken for 10 min. The separated MIBK layer was analyzed by atomic absorption spectrophotometry (Hitachi 170-50A).

Statistical Analysis All the values are expressed as mean±standard deviation of 6—8 rats. Significant differences between the mean values were determined by Student's *t*-test.

Results and Discussion

The curve of body weight did not significantly differ between copper deficient and pair-fed rats. The amount of ingested copper deficient diet per day gradually increased from 10 to 14 g during the experimental period.

As shown in the left panel of Fig. 1, copper concentrations in serum of rats at 2, 4, 6 and 8 d after they began feeding on a copper deficient diet were markedly reduced to approximately 62, 35, 20 and 16% of those of pair-fed rats, respectively. The copper concentration in liver of copper deficient rats was reduced with time after the administration of copper deficient diet, as shown in the right panel.

In rats given the copper deficient diet, a marked decrease of copper in the serum was found from early stages. This may show that the copper in serum was moving into the organs which severely require this ingredient. The liver, which contains many copper-enzymes, requires copper and apparently is not provided an adequate amount from the serum. Therefore, the copper in liver of rats given a diet deficient in copper may gradually decrease with time after the administration of this diet.

The subcellular distribution of copper in rat liver at 5 d after beginning the copper deficient diet is shown in Table I. The copper concentration in whole liver of normal rats or NDMA-treated rats given a copper deficient diet decreased to approximately 75% or 70% of pair-fed controls, respectively. The copper concentrations of soluble fraction and nuclear fraction decreased at a similar rate in copper deficient rats under the experimental conditions tested. No concentration of copper either in pair-fed rats or in copper deficient rats was altered by treatment with NDMA.

As shown in Fig. 2, the incorporation of [^{3}H]thymidine into liver DNA in NDMA-treated rats significantly increased approximately 4 times (left panel) and 6 times (right panel) more than that in pair-fed rats (not treated with NDMA). The increase of [^{3}H]thymidine incorporation into the liver DNA is considered to result from liver damage by NDMA which is an acute hepatotoxin. The

TABLE I. Subcellular Distribution of Copper in the Liver

Fraction	Copper concentration (μg/liver)			
	Pair-fed		Copper deficient	
	0.9% NaCl	NDMA[a]	0.9% Nacl	NDMA[a]
Whole liver	2.54±0.04	2.66±0.14	1.92±0.04[b]	1.89±0.08[b]
Nuclear	1.56±0.06	1.48±0.02	1.21±0.11[b]	1.03±0.07[b]
Mitochondrial	0.15±0.01	0.16±0.02	0.15±0.01	0.14±0.01
Microsomal	0.07±0.01	0.11±0.01	0.06±0.01	0.09±0.02
Soluble	0.76±0.05	0.92±0.12	0.50±0.08[c]	0.51±0.02[c]

The copper concentrations were measured at 5 d after starting a copper deficient diet. Each value represents the mean±S.D. of 6 rats. *a*) NDMA, *N*-nitrosodimethylamine. Significantly different from each fraction of pair-fed control, *b*) $p<0.05$, *c*) $p<0.01$.

TABLE II. Effect of Copper Deficiency on the Thymidine Kinase Activity in the Liver of Rats Treated with NDMA

Group	pmol TMP[a] formed/10 min/mg protein			
	5 d[b]		9 d[b]	
Pair-fed	6.5±0.8	(100)	5.6± 2.6	(100)
Copper deficient	6.1±0.3	(98)	7.7± 1.8	(137)
Pair-fed+NDMA	117.6±2.4[c]	(1897)	119.8± 7.1[c]	2139)
Copper deficient +NDMA	146.5±6.4[d]	(2363)	159.3±16.5[d]	(2845)

Each value represents the mean±S.D. of 6—8 rats. Figures in parenthesis are relative values. *a*) TMP, thymidine monophosphate. *b*) Time after starting the experimental diet. *c*) Significantly different from pair-fed group, $p<0.001$. *d*) Significantly different from pair-fed+NDMA group, $p<0.05$.

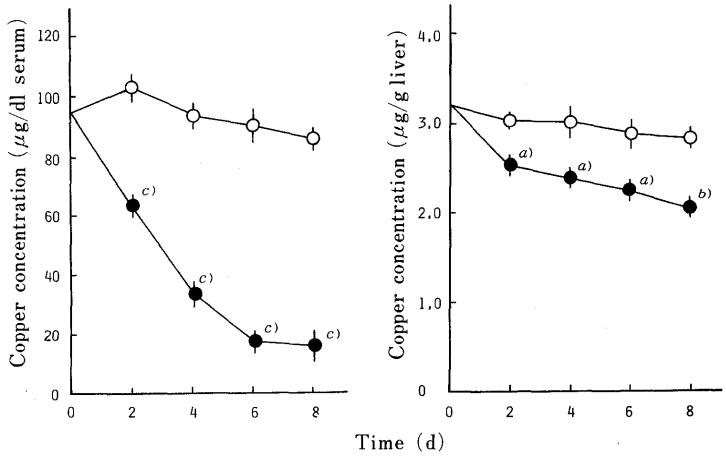

Fig. 1. Copper Concentrations in Serum (Left Panel) and Liver (Right Panel) of Copper Deficient and Pair-Fed Rats

For each point 6—8 rats were used. The symbols are as follows: —●—, copper-deficient rats; —○—, pair-fed rats. Significantly different from each of the pair-fed rats; *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.

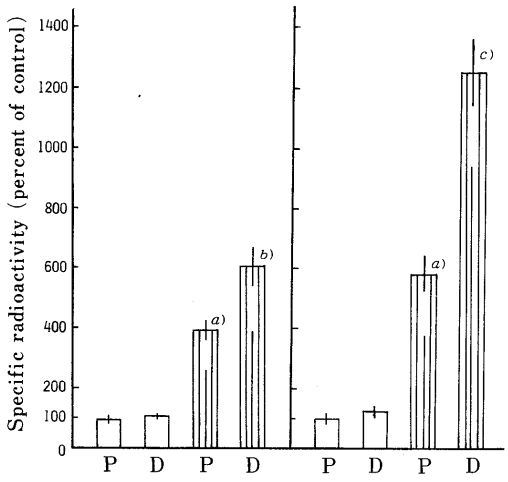

Fig. 2. Effect of Copper Deficiency on DNA Synthesis in the Liver of NDMA-Treated Rats

The values at 5 d (left panel) and 9 d (right panel) after starting to give rats the experimental diet are shown. For each group 7 or 8 rats were used. P, pair-fed rats; D, copper-deficient rats. The symbols are as follows: ⊓, normal rats; ⊓⊓, NDMA-treated rats. The values of control (pair-fed group of normal rats) are 4108 ± 370 dpm/mg DNA in the left panel and 3945 ± 769 dpm/mg DNA in the right panel. Significantly different from the pair-fed group, *a*) $p < 0.01$. Significantly different from the pair-fed group of NDMA-treated rats; *b*) $p < 0.05$, *c*) $p < 0.01$.

thymidine incorporation into the liver DNA in NDMA-treated rats was significantly enhanced by giving them a copper-deficient diet compared to pair-fed rats treated with NDMA. The thymidine incorporation into liver DNA of NDMA-treated rats at 5 and 9 d after starting them on a copper deficient diet increased to 153% (Fig. 2, left panel) and 214% (Fig. 2, right panel) that of pair-fed rats, respectively. The incorporation of [^{3}H]thymidine into liver DNA of rats administered a copper-deficient diet alone did not increase significantly. Therefore, even in the liver of animals given a normal diet copper may have a protective effect on potential liver damage by NDMA.

As shown in Table II, the activity of thymidine kinase in liver of pair-fed rats treated with NDMA significantly ($p < 0.001$) increased approximately 19 and 21 times more than that of pair-fed rats, at 5 and 9 d after beginning the experimental diet, respectively, while the activity in liver of copper deficient rats treated with NDMA significantly ($p < 0.001$) increased approximately 24 and 28 times at 5 and 9 d, respectively, after the diet was begun. The thymidine kinase activities of the copper deficient plus NDMA group significantly ($p < 0.05$) increased more than that of pair-fed plus NDMA group at both 5 and 9 d after diet introduction.

The mechanism by which copper deficiency leads to the enhancement of liver DNA synthesis in NDMA-treated rats should be studied further. A variety of abnormalities have been observed in rats fed a copper-deficient diet for a few weeks or months and these rats had severely low copper levels in serum and liver.[18-20] Under our experimental conditions tested, the copper concentrations in serum and liver of rats were reduced with time following administration of a copper-deficient diet. However, there was no significant difference in hepatic DNA synthesis between pair-fed and copper-deficient rats without treatment with NDMA (Fig. 2). It is of great interest that rats treated with NDMA during early stages of copper deficiency were observed to undergo an increase in DNA synthesis in the liver compared to pair-fed rats treated with NDMA, which may lead to greater hepatotoxicity and hepatocarcinogenicity.

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