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Enhancing Effect of a Choline-deficient Diet on Alterations of Hepatic Drug-metabolizing Enzymes in Hepatitis- and Hepatoma-predisposed Rats (LEC Rats)

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Marked alterations of hepatic drug-metabolizing enzymes were observed in hepatitis- and hepatoma-predisposed rats (LEC rats) fed a choline-deficient diet. The diet enhanced the development of hepatitis with severe jaundice. The levels of two major classes of cytochrome P-450, P-450$_{PB}$ and P-450$_{MC}$, were markedly decreased. GST-Yp was dramatically increased, whereas GST-Ya, Yb1 and Yb2 were decreased. LEA rats (the control rats to LEC) fed a choline-deficient diet mimicked LEC rats fed a normal diet in terms of the above enzyme alterations, indicating that hypomethylation is involved in the pathogenesis of hepatitis and hepatoma in LEC rats. Such hypomethylation may initiate the hepatocytes that spontaneously develop hepatitis and hepatoma.

Key words: Choline deficiency — Cytochrome P-450 — Glutathione *S*-transferase P — LEC rat — DNA-hypomethylation

LEC[2] rats are a mutant strain displaying a hereditary tendency to develop hepatitis with severe jaundice.[1] The hepatitis appears suddenly about three to four months after birth with a high rate of mortality. The frequency of appearance of jaundice in the rats is greater than 80%, and about 30% of the rats die of fulminant hepatitis within a week. Furthermore, the surviving rats spontaneously develop liver cancer with age. The genetic basis has been shown to be a single autosomal recessive mutation.[2,3] The mutation in the LEC rats is clearly different from that previously reported to cause mild hepatitis in Gunn rats, which are deficient in UDP-glucuronyltransferase,[4] or that causing hepatic cirrhosis, which is due to α_1-antitrypsin deficiency.[5] It is of interest to elucidate the biochemical factors involved in the pathogenesis of spontaneous hepatitis and subsequent liver cancer.

Previous studies[6,7] have shown that the composition and the activities of enzymes involved in drug metabolism in LEC rats are quite similar to those observed in hyperplastic foci and nodules induced by chemical carcinogens.[8,9] There are only a few appropriate experimental models for studying the relationship between the development of hepatitis and hepatocarcinogenesis. We thought choline deficiency might provide a useful model system. To study this relationship, it is important first to characterize choline-deficient diet-induced hepatitis in detail. In the present study, we used immunochemical and biochemical methods to investigate the hepatic enzyme alterations in LEC rats fed a choline-deficient diet.

MATERIALS AND METHODS

Chemicals NADPH and the choline-deficient diet devised by Mikol *et al.*[10] were obtained from Oriental Yeast Co., Japan. L-γ-Glutamyl-*p*-nitroanilide was from Tokyo Kasei Kogyo Co. Ltd., Japan. Polyclonal antibodies to glutathione *S*-transferase (GST) subunits, Yp, Ya, Yc, Yb1, and Yb2 were from BIOPREP (Dublin, Ireland). Horseradish peroxidase-conjugated anti-guinea pig IgG and anti-rabbit IgG were from Miles Yeda Ltd. Ethoxy- and pentoxyresorufin were synthesized by the method of Mayer *et al.*[11] All other reagents were of the highest grade commercially available. ^{32}P-dCTP was purchased from Amersham Corp. Plasmids containing cDNA probes for GST-P, β actin, and cytochrome P-450b, P-450c, and P-450d were gifts from Drs. M. Muramatsu (University of Tokyo), T. Noguchi (Osaka University), and M. Adesnik (New York University), and anti GST-P was from Dr. K. Sato (Hirosaki University).

Animals Male LEC (Long-Evans with a cinnamon-like coat color) and LEA (Long-Evans with an agouti coat color) rats were maintained at the Institute of Experimental Animal Sciences, Osaka University Medical School and at the Center for Experimental Plants and Animals, Hokkaido University, Sapporo. LEC and LEA rats were isolated from a closed colony of randomly bred Long-Evans rats, and LEA rats were used as a negative control, because these rats do not suffer from hepatitis.

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[2] The abbreviations used are: LEC, Long-Evans with a cinnamon-like coat color; LEA, Long-Evans with an agouti coat color; P-450$_{PB}$, phenobarbital-inducible P-450, mainly P-450b (P450IIB1) and P-450e (P450IIB2); P-450$_{MC}$, 3-methylcholanthrene-inducible P-450, mainly P-450c (P450IA1) and P-450d (P450IA2); GST, glutathione *S*-transferase.

There was no apparent sex difference in the appearance of hepatitis, and no apparent abnormalities (such as nodules) were observed by macroscopic assessment of livers from LEC rats during the experiments.

Young male LEC rats at 5 weeks, weighing from 60–80 g, and LEA rats at 5 weeks, weighing 90–110 g, were used in all the dietary experiments. Food and water were available *ad libitum* unless otherwise noted. Four groups of 15 animals each were fed one of two diets and were killed after 4 weeks.

Tissue preparation Animals were killed by decapitation, and the livers were perfused with cold physiological saline (0.86% NaCl) and homogenized in 5 volumes of cold 0.25 *M* sucrose containing 5 m*M* Tris-HCl, pH 7.4, and 1 m*M* EDTA with a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 9,000*g* for 15 min, and the resulting postmitochondrial supernatant fraction was centrifuged at 105,000*g* in a Hitachi RP-55T rotor for 1 h. The pellet was resuspended in 50 m*M* potassium phosphate, pH 7.4. The microsomal and the cytosolic fractions were stored at −80°C for up to one month until analysis.

Analytical methods The contents of cytochrome P-450 and cytochrome b_5 were measured spectrophotometrically by the method of Omura and Sato.[12] The activities of hepatic microsomal drug-metabolizing enzymes such as γ-glutamyltranspeptidase (γ-GTP), glutamic oxaloacetic transaminase (GOT), and GST were measured according to methods described previously.[6] Ethoxyresorufin *O*-deethylase and pentoxyresorufin *O*-dependentylase activities were assayed by the method of Lubet *et al.*[13] Briefly, reaction mixtures contained 0.5 ml of 50 m*M* Tris-acetate, pH 7.5, 25 m*M* $MgCl_2$, 2 μ*M* ethoxyresorufin or 2 μ*M* pentoxyresorufin, and 30 μl of microsomes (0.5–0.6 mg of protein). The reaction was initiated by the addition of NADPH (final concentration, 0.2 m*M*) and monitored by recording the increase in relative fluorescence of the product (resorufin) at 30°C. Protein was determined by the procedure of Lowry *et al.*[14] with bovine serum albumin as standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted in 8% and 15% gels for P-450 and GST, respectively, according to the method of Laemmli.[15] Western blotting was carried out using polyclonal antibodies against $P\text{-}450_{PB}$, $P\text{-}450_{MC}$, or GST as described previously.[6]

Extraction of RNA and Northern hybridization The total cellular RNA from the liver was extracted by a single-step guanidium hydrochloride method. Poly-$(A)^+$ RNA was purified by oligo (dT) column chromatography.[16] Northern blots were hydridized under stringent conditions (50% formamide and 4× stock solution) at 42°C for 24 h, using 5×10^7 cpm per blot. The filters were washed to a final stringency of 0.1× stock solution and 0.1% SDS at 45°C, and exposed to Kodak X-ray film at −70°C with intensifying screens.

RESULTS

Growth and hepatitis The choline-deficient diet led to weight decreases in both LEC and LEA male rats. No significant growth was seen in rats fed this diet (Fig. 1). Six out of fifteen LEC rats died between experimental weeks 3 and 4, and more interestingly, four of them were found to have jaundice. The incidence of hepatitis in LEC rats fed a normal diet closely resembled that seen in previous work.[1] On the other hand, all of the LEA rats were still alive without any significant clinical symptoms after 4 experimental weeks. There was thus a clear association between the choline-deficient diet and a high incidence of jaundice only in the LEC rats. The choline-deficient diet exerted a pronounced effect on the relative risk for jaundice in the LEC rats.

Hepatic drug-metabolizing enzymes in LEC and LEA rats fed a choline-deficient diet Table I lists the changes in the hepatic drug-metabolizing enzymes in LEC and LEA rats after 4 weeks on a choline-deficient diet. Total microsomal cytochrome P-450 levels were decreased to about 35% of the control in LEC rats. Ethoxyresorufin *O*-deethylase and pentoxyresorufin *O*-dependentylase activi-

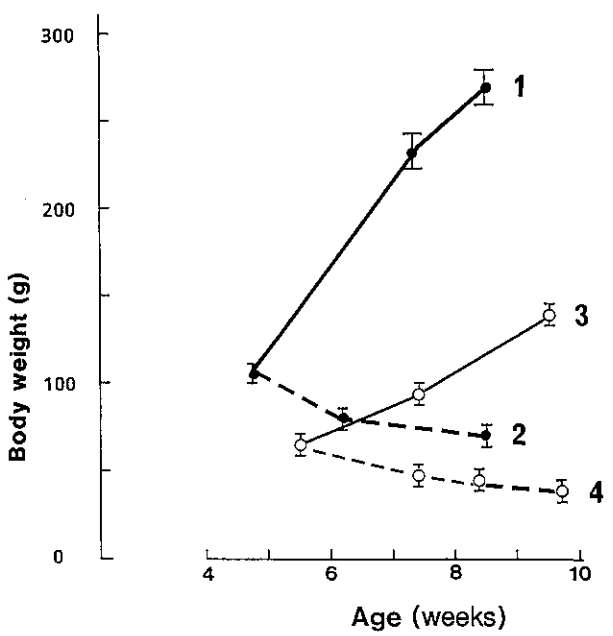

Fig. 1. Effects of a choline-deficient diet on the growth of LEC and LEA male rats. 1, LEA control diet; 2, LEA choline-deficient diet; 3, LEC control diet; 4, LEC choline-deficient diet. Points, mean of 9 to 15 values; bar, SD.

Table I. Effect of Choline Deficiency (CD) on Contents and Activities of Drug-metabolizing Enzymes in LEC and LEA Male Rats

	LEC		LEA	
	Control diet	CD diet	Control diet	CD diet
Cytochrome P-450[a]	0.81±0.23 (100)	0.28±0.04 (35)*	1.33±0.02 (100)	0.52±0.02 (39)**
Cytochrome b_5[a]	0.49±0.12 (100)	0.19±0.02 (39)*	0.50±0.02 (100)	0.27±0.04 (56)**
Ethoxyresorufin *O*-deethylase[b]	18.2±2.7 (100)	2.48±0.54 (14)**	21.2±1.8 (100)	21.9±2.7 (103)
Pentoxyresorufin *O*-depentylase[b]	4.46±0.94 (100)	0.59±0.33 (13)**	26.3±1.8 (100)	6.06±0.48 (23)**
γ-Glutamyltranspeptidase[c]	2.38±0.66 (100)	13.5±1.6 (567)**	1.33±0.07 (100)	12.8±1.9 (960)**
Glutathione *S*-transferase[d]	0.75±0.11 (100)	0.36±0.09 (48)*	1.52±0.05 (100)	0.72±0.07 (47)**
Glutamic oxaloacetic transaminase[e]	159±36 (100)	197±34 (122)	105±26 (100)	267±50 (254)**

a) nmol/mg protein; *b*) pmol/min/mg protein; *c*) nmol/min/mg protein; *d*) μmol/min/mg protein; *e*) nmol/min/ml serum. Values are mean±SD of five individual livers in each group. Numbers in parentheses indicate the percentage of the control for each rat. Significantly different from control value in Student's *t* test: *, $P<0.05$; **, $P<0.01$.

ties were markedly decreased to 14% and 13% of the control, respectively. In contrast, γ-glutamyltranspeptidase was dramatically increased to 567% of the control level. A small elevation of serum glutamic oxaloacetic transaminase was observed in LEC rats fed the choline-deficient diet, suggesting the presence of liver cell injury. The choline-deficient diet decreased the cytosolic GST activity of LEC rats to about 48% of the control, when 1-chloro 2,4-dinitrobenzene (CDNB) was used as a substrate.

The effects of choline-deficient feeding on the activities of these enzymes in LEA rats were quite similar to those observed in LEC except for ethoxyresorufin *O*-deethylase activity. The P-450 content, pentoxyresorufin *O*-depentylase activity and GST activity decreased to 39%, 23%, and 47% of the levels in control LEA rats, respectively, whereas ethoxyresorufin *O*-deethylase activity remained the same as in LEA control rats. γ-Glutamyltranspeptidase activity increased to almost the same level as in LEC rats fed the choline-deficient diet.

Western blot analysis of P-450 and GST isozymes in LEC rats fed the choline-deficient diet Immunoquantitations of hepatic drug-metabolizing enzymes were carried out. Levels of the $P\text{-}450_{PB}$ and $P\text{-}450_{MC}$ forms of cytochrome P-450 were markedly decreased in liver microsomes from LEC rats fed the choline-deficient diet (Fig. 2, lanes 3 and 4 in panel A ($P\text{-}450_{PB}$) and lanes 3 and 4 in panel B ($P\text{-}450_{MC}$)). The cytochrome $P\text{-}450_{PB}$ level was only 35% of that in control microsomes, and the $P\text{-}450_{MC}$ level was decreased to 16% of the control (Fig. 3).

GST is classified into three subfamilies, α, μ, and π.[17] Polyclonal antibodies raised against rat GST can be used to distinguish five different isozymes. Fig. 2, lanes 3 and 4 in panel C shows a typical immunoblot for GST-P from an LEC rat fed a choline-deficient diet. GST

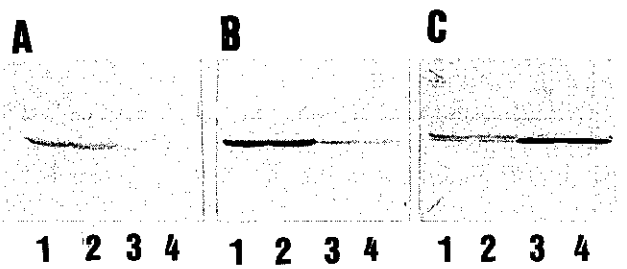

Fig. 2. Western blot analysis of cytochromes $P\text{-}450_{PB}$ (A) and $P\text{-}450_{MC}$ (B), and GST-P (C) in LEC rats fed a choline-deficient diet. Immunoblots were carried out using 40 μg of protein per lane for each microsomal preparation (panels A and B), and 20 μg of protein per lane for each cytosolic one (panel C). Lanes 1 and 2, control diet; lanes 3 and 4, choline-deficient diet.

contents of individual livers from LEC rats were quantified by Western blotting (Fig. 3). There was a marked difference in the relative composition of GST subunits between rats fed the choline-deficient and the control diets. Following choline-deficient diet feeding, LEC rats had 26-fold higher levels of GST-Yp protein than LEC rats fed the control diet. In contrast, the levels of GST-Ya, Yb1 and Yb2 decreased to 59, 77, and 42% of the control, respectively.

Northern blot analysis of P-450 isozymes and GST-P in LEC rats fed the choline-deficient diet Northern blots showed that the expression of P-450b, P-450c, and P-450d mRNA in LEC rats fed the choline-deficient diet was markedly suppressed to 14, 1, and 4% of the control, respectively, whereas a 3.8-fold induction of GST-P mRNA was seen in the LEC rat livers following choline-deficient diet feeding (Fig. 4).

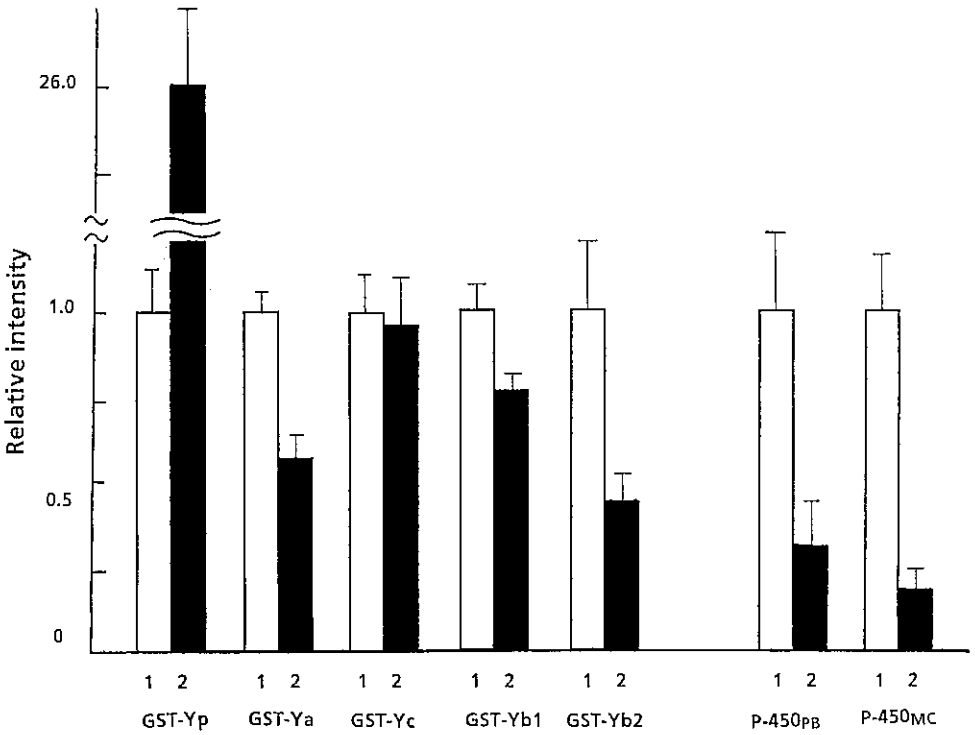

Fig. 3. Relative quantitation of GST subunits, and cytochromes P-450$_{PB}$ and P-450$_{MC}$ in LEC rats fed control diet and choline-deficient diet. The columns represent the means of triplicate samples, and the bars, SD. 1, control diet; 2, choline-deficient diet.

DISCUSSION

In this study, we demonstrated that cytochrome P-450 in hepatic microsomes from LEC and LEA rats fed a choline-deficient diet has a reduced capacity to catalyze the oxidation of pentoxyresorufin as compared with ethoxyresorufin. Similar selective changes in other mixed function oxidase activities may occur in spontaneous hepatic carcinogenesis of LEC rats.[6] At 4 weeks the cytochrome P-450 content of the LEC rat liver was 43% of the control (LEA) value, and at 3 months the level was 65% of the control. Takahashi *et al.*[18] have reported that the liver of the LEC rat was highly sensitive to carcinogenic stimulation by diethylnitrosamine, and we[7] have also reported that drug-metabolizing enzymes in the LEC rat were induced by phenobarbital and methylcholanthrene, as in LEA rat.

It is well-known that changes in the levels and activities of cytochrome P-450 isozymes occur following exposure to xenobiotics.[19, 20] It has also been shown that pathological and nonphysiological states such as hepatitis,[21, 22] hepatoma,[23] jaundice,[24] and various other pathological conditions[25-27] can influence the level of total cytochrome P-450 as well as microsomal monooxygenase activities. Murray *et al.*[26] have demonstrated that hepatic cirrhosis resulting from prolonged intake of a choline-deficient diet impaired the regulation of male-specific P-450 isozymes, whereas levels of P-450$_{PB\text{-}c}$ (P450IIC6), P-450$_{ISF\text{-}G}$ (P450d, P450IA2) and female-specific P-450$_{UT\text{-}I}$ (P450IIC12) were apparently un-

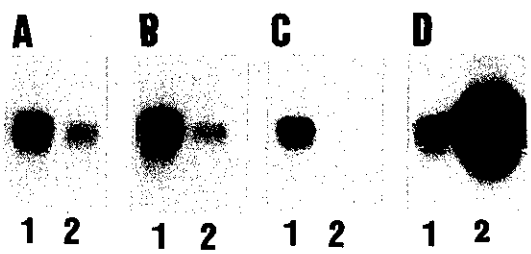

Fig. 4. Northern blot analysis of hepatic mRNA coding for cytochromes P-450b (panel A), P-450c (panel B), and P-450d (panel C), and GST-P (panel D) from LEC rats fed control diet (lane 1) and choline-deficient diet (lane 2). Northern blot analysis was carried out with 3 μg of hepatic poly(A)$^+$-rich RNA obtained by purification on poly (dT) Sepharose. The agarose gel electrophoresis was performed as described under "Materials and Methods."

altered. In this study, the level of P-450$_{PB}$ in LEA rats fed a choline-deficient diet was greatly decreased to less than 23% of the LEA control in proportion to the decrease in total microsomal P-450. The level of P-450$_{MC}$, associated with ethoxyresorufin *O*-deethylase activity, underwent no significant change in the LEA rats.

GST 1-2, 2-2, and 7-7 are known to possess Se-independent GSH-peroxidase activity toward lipid peroxides. Kitahara *et al.*[28] have observed increased GST-A (3-3) in rat preneoplastic hepatic lesions induced by chemical carcinogens. GST-P (7-7) may be related to prevention of cytotoxic lipid peroxidation, which plays an important role during tumor promotion by a choline-deficient diet. Compared with other isozymes, GST-P is less sensitive to inhibition by endogenous substances such as bilirubin, hematin, or bile acids, and therefore increased activity of GST-P in preneoplastic foci is thought to be a favorable adaptation rendering the cells resistant to cytotoxic agents.[17] In the case of LEC rats, even the 26-fold increase in GST-P content seen in the present study probably provides insufficient protection against the excess hydrogen peroxide produced by peroxidation due to choline deficiency, given the decrease in total GST activity.

It is of interest that GST-P is expressed not only in putative preneoplastic foci induced by chemicals but also in spontaneous lesions of "altered cell foci" in LEC rats.[29,30] GST-P-positive foci appeared in the livers of 5-month-old LEC rats, and the number of rats affected and the size of the foci increased with age. Such a high incidence of spontaneously occurring foci in young rats is quite rare in other strains. Moore *et al.*[31] and Yokota *et al.*[32] observed some single GST-P-positive hepatocytes in the livers of control animals, without exposure to any chemical carcinogens. These may represent putative initiated hepatocytes (endogenously initiated cells) which progress to hepatoma. In the present study, the increase in GST-P expression in preexisting GST-P-positive hepatocytes of young LEC rats was striking after the start of the choline-deficient diet.

A choline-deficient diet appears to strongly enhance hepatitis development in LEC rats. The rather short-term (4 weeks) administration of a choline-deficient diet produced a high incidence of hepatitis in the LEC rats. Severe jaundice had developed in two-thirds of the dead rats. In this study we found that a choline-deficient diet caused changes in the hepatic drug-metabolizing enzymes in both LEC and LEA rats similar to those occurring spontaneously in LEC rat. Fig. 5 shows a comparison of the enzyme alterations in the choline-deficient diet-fed LEA and the normal diet-fed LEC rats. The similarity of the pattern suggests that LEA rats fed a choline-deficient diet mimic LEC rats and that LEC rats have endogenously initiated cells which are spontaneously

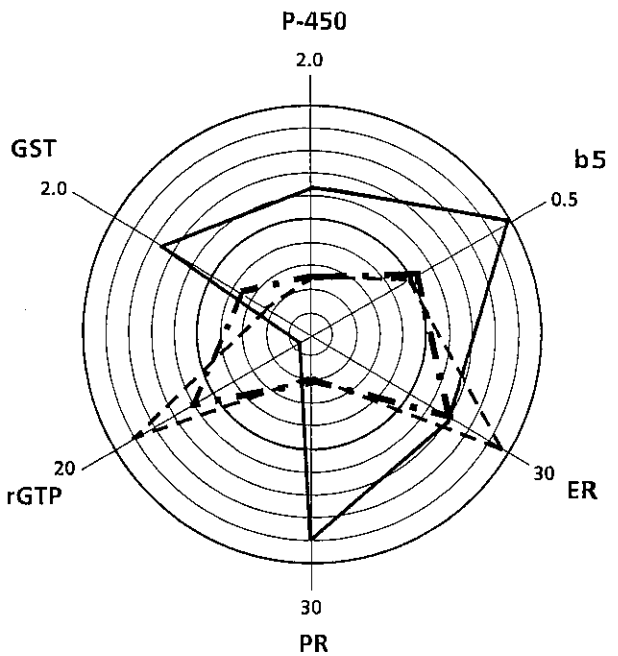

Fig. 5. Comparison of the hepatic drug-metabolizing enzymes of LEA rats fed control diet (——), LEC rats fed control diet (---), and LEA rats fed choline-deficient diet (—·—). b_5, cytochrome b_5; ER, ethoxyresorufin *O*-deethylase; PR, pentoxyresorufin *O*-dependentylase; γGPT, γ-glutamyltranspeptidase.

developing hepatitis and hepatoma. These findings tend to support the belief that the LEC rats have a biochemical predisposition to hepatitis. This is the first demonstration of common cellular responses to a methyl-group-devoid diet and endogenous factors acting as inducers of hepatitis.

Certain dietary modifications lead to alterations in cells increasing their susceptibility to carcinogens. Thus, choline deficiency in rats has been shown to enhance the induction of preneoplastic foci as well as hepatoma by well-known carcinogens. Rushmore *et al.*[33] demonstrated that a choline deficiency leads to increased lipid peroxidation in nuclei and this in turn leads to "damage" to DNA followed by cell death and cell proliferation. This hypothesis of DNA alterations coupled with regenerative hepatocyte proliferation is attractive to explain the initiation of hepatocarcinogenesis in rats fed a choline-deficient diet.

As described above, LEA rats fed a choline-deficient diet mimic LEC rats, indicating that LEC rats have some defects in methyl-group metabolism including DNA hypomethylation. It is noteworthy that a co-ordinate change of enzyme pattern occurs during chemical carcinogenesis in preneoplastic foci, and in hepatic tissue

after a choline-deficient diet. An attractive regulatory mechanism for the pathogenic co-ordinate change is proposed to be DNA hypomethylation. In fact, changes of NADPH quinone oxidoreductase[34] and P-450[35] have recently been shown to be related to hypomethylation during the development of preneoplastic nodules or aging. Our observations in rats with dietary methyl-group deficiency provide further support for the involvement of hypomethylation.

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Addendum: In a very recent study by Li *et al.*,[36] jaundiced autosomal recessive LEC rats were found to have a genetic defect in copper metabolism resulting in a severe accumulation of copper and an elevated level of metallothionein in the liver, and a negligible level of serum ceruloplasmin. These symptoms closely resemble those in humans with Wilson's disease. The LEC rat is therefore a useful animal model for this disease.

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