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# Hepatocellular Carcinoma Induction in LEC Rats by a Low Dose of 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline

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**A food-borne heterocyclic amine, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), induces hepatocellular carcinomas (HCCs) in F344 male rats at an incidence of 95%, when fed in the diet at 400 ppm for 61 weeks. In this study, the effect of a low dose of MeIQx was examined in Long-Evans with cinnamon-like coat color (LEC) rats, which have a mutation in *Atp7b* and suffer from hereditary hepatitis and HCCs, with high levels of copper accumulation in the liver. Rats of the LEC and Long-Evans with agouti coat color (LEA) sibling lines were given a diet containing 40 ppm MeIQx from the age of 23 weeks to 63 weeks, for a total administration period of 40 weeks. In LEC rats, HCCs were observed in 8/8 animals administered MeIQx, and 2/8 rats receiving a normal diet. The number of HCCs per rat (mean±SD) was 2.8±2.0 and 0.3±0.5, respectively. In the LEA rats, however, no tumors were induced by administration of MeIQx. These results indicate that damaged liver associated with compensatory cell proliferation is much more susceptible to chemical hepatocarcinogens, including MeIQx, than the normal liver.**

Key words: MeIQx — LEC rat — Hepatocellular carcinoma — Copper

Heterocyclic amines generated in cooked food have been shown to be genotoxic in short-term tests and carcinogenic when given long-term to rodents,[1–3] and also to monkeys.[4] Dietary supplementation with 600 ppm of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), one of these important environmental contaminants, induced liver tumors in 91% and lung tumors in 43% of female treated mice, and liver tumors in 43% and leukemia and lymphomas in 29% of males, within 84 weeks.[5] In the rat, at 400 ppm in the diet, it induced tumors in the liver (100% in males and 53% in females), skin (35% in males), Zymbal gland (75% in males and 53% in females) and clitoral glands (63% in females).[6] While the degree of actual exposure to this chemical in our daily lives is calculated to be about 1/1000 or 1/10000 of the doses used in animal carcinogenicity experiments, as assessed by measuring its presence in the food or urine samples of healthy volunteers,[7,8] the risks of this compound in human carcinogenesis are unknown. We previously found that chronic administration of MeIQx at doses of 0.4 to 400 ppm led to the formation of DNA adducts in a linear dose-dependent manner in rat liver.[9] This suggests that even at very low doses in the range of human consumption, heterocyclic amines can generate DNA adducts which may result in genetic alteration, leading to cancer development, directly or indirectly. It has not yet been proven whether heterocyclic amines cause human cancer or not, but they certainly have been implicated as playing a possible role in one or more of the multi-steps involved in human carcinogenesis.

Long-Evans with cinnamon-like coat color (LEC) rats have been established as a mutant strain characterized by hereditary hepatitis and high yields of 'spontaneous' hepatocellular carcinomas (HCCs).[10–13] Hepatitis with jaundice in LEC rats develops at about 18–20 weeks of age, associated with very high levels of serum glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT), and then becomes chronic. HCCs appear from about 60 weeks of age and a 100% incidence has been observed within 90 weeks.[11,13] Cholangiofibrosis also develops with aging in the same manner.[11,13] High levels of copper have been reported in the livers of LEC rats[14,15] and a hereditary linkage between this copper accumulation and hepatitis development has been demonstrated, suggesting that the liver damage is in fact directly due to excess copper.[16] It has been revealed that the LEC rats have a deletion mutation in the *Atp7b* gene,[17] a human homologue of which is involved in Wilson's disease.[18–20]

Development of hepatocellular carcinomas in humans is closely associated with liver cirrhosis induced by hepatitis virus infection or the heavy consumption of alcoholic beverages.[21–23] Wilson's disease patients also develop liver cancer, although the number of reported cases

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Table I. Data of Final Body Weight, Final Liver Weight, Plasma GPT and Copper Contents

| Group | Strain/ treatment | Effective no. of rats | Body wt. (g) | Liver wt. per 100 g body wt. (g) | Plasma GPT (IU/liter/37°C) | Amounts of copper | |
|---|---|---|---|---|---|---|---|
| | | | | | | Plasma ($\mu$g/ml) | Liver[a] ($\mu$g/g) |
| 1 | LEC/untreated | 8 | 342±13 | 3.66±0.36 | 117±29 | 0.48±0.08 | 349±147 |
| 2 | LEC/MeIQx | 8 | 314±24[b] | 5.36±0.83[c] | 117±18 | 0.34±0.09 | 328±160 |
| 3 | LEA/MeIQx | 8 | 575±19 | 3.52±0.32 | 46±12 | 1.21±0.12 | 5±0 |

Values are means±SD.
*a*) Non-tumorous portions of the liver.
*b*), *c*) Significant difference at $P<0.05$ and $P<0.001$, respectively, between groups 1 and 2.

Table II. Hepatic Histological Findings

| Group | Effective no. of rats | No. of rats with hepatocellular lesions | | No. of HCC/rat[a] | No. of rats with other lesions | |
|---|---|---|---|---|---|---|
| | | Adenoma | Carcinoma | | Cholangio-fibrosis | Cystic adenoma |
| 1 | 8 | 5 | 2 | 0.3±0.5 | 5 | 0 |
| 2 | 8 | 4 | 8 | 2.8±2.0[b] | 4 | 4 |
| 3 | 8 | 0 | 0 | 0 | 0 | 0 |

*a*) Values are means±SD.
*b*) Significant difference from group 1 at $P<0.05$.

is small.[24] Therefore, livers with hepatitis cirrhosis might be more susceptible to low doses of environmental chemical carcinogens than normal livers.

Thus, we examined the effects of a low dose of MeIQx on hepatocarcinogenesis in LEC rats, administering the compound during the chronic hepatitis phase.

## MATERIALS AND METHODS

MeIQx was purchased from the NARD (Osaka). The purity of this compound was more than 99% by HPLC analysis monitored with A260. A pellet diet manufactured by adding MeIQx to a basal diet (CE-2) was supplied by CLEA Japan, Tokyo.

LEC and LEA (Long Evans with agouti coat color) male rats at 6 weeks of age were obtained from the Center for Experimental Plants and Animals, Hokkaido University, and then maintained in our laboratory. LEA, a sibling line of LEC, which does not suffer from liver disease, was used as a control. The animals were housed 3 or 4 per cage in an air-conditioned room at 22±2°C. Sixteen 23-week-old LEC rats were divided into 2 groups of 8 animals, one being given a CE-2 diet (group 1), and the other the pellet diet containing MeIQx at 40 ppm (group 2) for 40 weeks. Eight LEA rats were given 40 ppm MeIQx in the same manner (group 3). Animals were killed 40 weeks after the start of the experiment (at 63 weeks of age) and their blood was sampled. GPT levels in the plasma were determined with an auto-analyzer (Hitachi 736). Livers were excised and examined for gross lesions. Tumorous portions of the liver were fixed in 10% phosphate-buffered formalin solution and processed for histological examination according to the criteria reported previously.[25]

For measurement of copper concentration, liver tissues and plasma samples were kept at −80°C until use. Samples of 30–70 mg of the tissue or 50 $\mu$l of plasma were mixed with 1 ml of nitric acid (sp.gr. 1.42) and heated, first at 90°C for 1–2 h and then at 110°C for 10 h in acid-washed glass tubes. Ashed samples were dissolved in 2 ml of 0.1 *N* nitric acid, and copper concentrations in the liver were determined by inductively coupled plasma (ICP) emission spectroscopy (Plasma Spec; Leeman Laboratories, Inc., Boston, MA), and in the plasma, by flameless atomic absorption spectroscopy (Hitachi Co., Tokyo).

## RESULTS

Data on the final body weights, final relative liver weights and plasma GPT levels, as well as plasma and hepatic copper concentrations are summarized in Table I. The body weights of MeIQx-administered LEC rats (group 2) were lower than in the LEC controls (group 1). In contrast, the relative liver weights in group 2 were 46% higher than those of group 1. The plasma GPT levels, and plasma and liver copper concentrations did not differ between groups 1 and 2. The plasma GPT

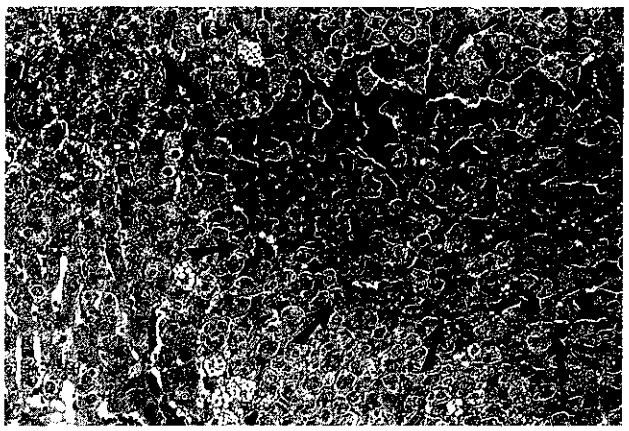

Fig. 1. Hepatocellular adenoma in an untreated LEC rat. The lesion exhibits obvious compression of the surrounding non-adenomatous area. Hematoxylin and eosin stain. ×50 (original magnification).

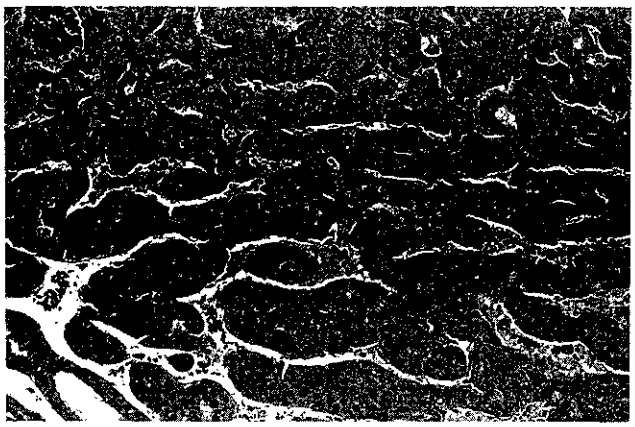

Fig. 2. Hepatocellular carcinoma in an LEC rat given MeIQx. The lesion shows trabecular formation and cellular atypia is evident. Hematoxylin and eosin stain. ×50 (original magnification).

levels, and plasma and liver copper concentrations were essentially the same in MeIQx-treated LEA (group 3) as the reported values for untreated animals of this strain.[26] The average carcinogen intake of rats given 40 ppm MeIQx was 780 μg/day/rat in LEC rats (group 2) and 840 μg/day/rat in LEA rats (group 3).

Data for hepatic lesions observed in the LEC rats are summarized in Table II. The histological features of hepatocellular adenomas in groups 1 and 2 were the same as those illustrated in Fig. 1. Most of them were associated with an appearance of peliosis hepatis, being soft, reddish, and closely resembling the surrounding hepatic cells (not shown). Areas of cholangiofibrosis were evident in both groups 1 and 2. In contrast, cystic adenomas with proliferation of epithelial cells derived from bile ducts were only observed in group 2.

All LEC rats in the MeIQx-treated group (group 2) developed HCCs, whereas malignant liver lesions were found in only 2 of 8 rats in the untreated group (group 1). All the HCCs were diagnosed as well-differentiated carcinomas (Fig. 2). The number of HCCs/rat in group 2 was $2.8 \pm 2.0$, being significantly higher than that, $0.3 \pm 0.5$, in group 1 ($P < 0.05$).

General observation did not reveal any evidence of tumors in organs other than the liver. No tumors developed in LEA rats fed an MeIQx diet.

## DISCUSSION

In the present study, administration of MeIQx at a low dose (40 ppm), only 1/10 of that known to induce liver cancer in F344 rats, significantly enhanced the development of HCCs in LEC rats while not causing any preneoplastic or neoplastic lesions to the liver in the sibling LEA line. Since no HCCs were induced in F344 rats receiving 100 ppm of this compound in the diet for 52 weeks,[27] the present findings strongly indicate that the damaged liver with associated compensatory cell proliferation, is highly susceptible to MeIQx carcinogenicity. Although a high susceptibility to diethylnitrosamine, benzo[*a*]pyrene, *N*-methyl-*N*-nitrosourea and 2-acetylaminofluorene was observed in LEC rats using glutathione S-transferase placental form (GST-P)-positive focus formation as an end point, with the modified protocol of Solt and Farber,[28] we have, in this study, demonstrated for the first time the induction of HCC in LEC rats using a carcinogen at a dose that is non-carcinogenic in F344 rats.

Our previous study demonstrated that the level of MeIQx-DNA adducts in cirrhotic livers induced by a choline-deficient diet or injections of carbon tetrachloride was lower than in the normal liver after administration of an MeIQx diet.[29, 30] Cell proliferation is increased in these liver lesions, and this is likely to have played a key role in sensitizing cells to the low levels of DNA adducts formed by the carcinogen. Labeling indices in the chronic phase of hepatitis in LEC rats are about 4% at 28 weeks of age and 0.2% at 63 weeks of age,[31, 32] while those in LEA rats are less than 0.1% at all ages over 14 weeks.[31]

In the LEC strain, it is possible that copper acts as a co-carcinogen with MeIQx or other carcinogens since it is known that copper in nuclei is bound to DNA or matrix proteins.[33–36] Furthermore, copper was also found to induce active oxygen species, under conditions where $H_2O_2$ or ascorbic acid was present,[37] and to be mutagenic.[38, 39] Thus, in LEC rats, active oxygen radicals and/or copper itself could be involved in hepatitis devel-

opment at a young age and hepatic tumor development later. Actually, the level of 8-hydroxyguanine in the liver DNA of LEC rats is higher than in LEA rats.[40] We have found that MeIQx induces hepatic cell proliferation in F344 rats in a linear dose-dependent manner (unpublished results). MeIQx forms DNA adducts in various organs, and its tissue specificity in terms of carcinogenesis is considered to be delimited by the cell proliferation induced. Under conditions where cell proliferation is an *a priori* factor, various types of low-dose DNA-damaging agents could therefore be carcinogenic. This is of especial significance in the case of heterocyclic amines which humans consume on an almost daily basis.

It has recently been found that LEC rats have another gene determining high susceptibility to hepatocarcinogenesis. The trait of high susceptibility was dominant and independent of copper accumulation.[41] This genetic trait should also be connected to the high susceptibility of LEC rats to low doses of MeIQx.

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