Chronic liver injury promotes hepatocarcinogenesis of the LEC rat

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The Long–Evans rat with a cinnamon-like color (LEC) is a mutant rat that spontaneously suffers from chronic liver injury and subsequent hepatocellular carcinoma (HCC) caused by abnormal copper accumulation in the liver. We attempted to elucidate the role of prolonged liver cell injury on LEC rat hepatocarcinogenesis using a copper-deficient diet (CuDD) to inhibit the occurrence of consequent liver injury. The animals were fed the CuDD from the age of 4 weeks until being killed at the age of 10 months. Diethylnitrosamine (DEN) was administered at the age of 8 weeks. Groups fed a basal diet (BD) with or without the administration of DEN were also assigned as control groups. The animals fed the BD manifested liver injury, while those fed the CuDD did not show liver dysfunction until death.

Regarding the size of the lesions, which indicated the intensity of the promotive effect, the lesions in the livers of rats fed the BD with DEN were much larger than those of rats fed the CuDD with DEN. Feeding the LEC rats with CuDD completely suppressed the manifestation of liver injury, and it was clearly shown that prolonged liver injury had a promotive effect on the LEC rat hepatocarcinogenic process.

Introduction

The Long–Evans rat with a cinnamon-like color (LEC*) is a novel mutant rat that spontaneously suffers from chronic liver injury and subsequent hepatocellular carcinoma (HCC). Approximately 30–40% of LEC rats die at around the age of 4 months from acute liver failure, the remaining rats survive with chronic hepatitis and develop preneoplastic and neoplastic liver lesions. More than 1.5 years after birth almost 100% of the rats of both sexes manifest liver cancers (1). It was found that abnormal copper accumulation occurs in the liver of the LEC rat (2) and that this is linked genetically to the occurrence of liver injury (3). Recently the gene involved with the abnormal copper accumulation was reported to correspond to a rat homolog of the Wilson’s disease gene (4–6).

Unlike the patients of Wilson’s disease, LEC rats suffering from chronic liver injury manifest hepatocellular carcinomas (HCCs) with age. Similarly, in humans an increased incidence of hepatoma is reported to be associated with the chronic hepatitis and cirrhosis produced by the hepatitis B virus (7). Thus, the LEC rat is regarded as a good animal model for the investigation of liver injury and subsequent HCC development. On the other hand, there are a number of reports experimentally researching the relationship between liver injury and HCC development. Recent studies have attempted to simulate the process of the liver injury and HCC development using transgenic mouse systems introduced by oncogenes (8), hepatitis B viral genomes (9,10), and Z#2 α1-antitrypsin (11). They showed that continuous compensatory cell proliferation caused by cell injury and the resulting loss of cells have important effects on the development of HCC.

It was reported that administration to LEC rats of a copper-chelating agent, D-penicillamine, which reduces the abnormal copper accumulation in the liver, prevents the development of hepatitis (12) and spontaneous HCC (13). In this study LEC rats were fed a copper-deficient diet or basal diet containing a normal level of copper and administered the hepatocarcinogen diethylnitrosamine (DEN) to induce preneoplastic liver lesions.

The number and area of preneoplastic lesions were measured to examine the promotive effect of chronic liver injury on the development of HCC, showing that chronic liver injury significantly promoted the development of such lesions.

Materials and methods

Experimental protocols

Male 4-week-old LEC rats were purchased from Charles River Japan Inc. (Hino, Japan). They were maintained in rooms with temperature and light control until being killed. They were divided into three groups: a group fed a basal MF diet (BD) from Oriental Yeast Co. (Tokyo, Japan) without any treatments (group BD, n = 21), a group fed the BD with a single i.p. injection of 20 mg/kg of DEN at 8 weeks of age (group BD + DEN, n = 20), and a group fed a synthetic copper-deficient diet (CuDD) from Clea Japan Inc. (Tokyo, Japan) with a single i.p. injection of 20 mg/kg of DEN at 8 weeks of age (group CuDD + DEN, n = 9). CuDD was AIN–76 diet (14) in which the concentration of copper was greatly reduced. Copper content in BD and CuDD was ~7.2 mg/kg and 0.05 mg/kg, respectively. The animals were allowed free access to food and water. At the age of 10 months the animals were killed under ether anesthesia followed by the removal of the livers. The liver tissues were sliced and fixed in 10% formalin for hematoxylin and eosin (H&E) staining and immunohistochemical staining for glutathione S-transferase placental form (GST-P). A part of the liver tissue was frozen in liquid nitrogen and stored at −80°C until later use in biochemical examinations.

Evaluation of hepatitis

The levels of plasma glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and copper in each animal were measured at death. In addition, the time course of the changes of plasma GOT, GPT and copper levels in some of the animals fed the BD or CuDD were also monitored at the ages of 2, 4, 5, 6, 7, 8, 9 and 10 months. Microscopical examinations of the liver sections with H&E staining were performed to assess the development of hepatitis. In addition, mitotic figures of hepatocytes were counted.
Analyses of preneoplastic liver lesions

To analyze the preneoplastic lesions, immunohistochemical staining for GST-P was performed. Liver sections 4–6 µm thick were incubated with polyclonal rabbit anti-rat GST Yp (Boehringer Mannheim) for 1 h at room temperature. After several washes with PBS, the sections were incubated with the second antibody, peroxidase-conjugated goat anti-rabbit IgG (Dako A/S, Glostrup, Denmark, 1:100 dilution), for 1 h at room temperature. Then the slides were incubated in 0.04% diaminobenzidine solution.

The number (cm²) and the size of each intersection of GST-P-positive foci (>0.002 mm²) on the liver sections were measured using the image-analyzing program NIH Image on a Macintosh Quadra 800 computer (Apple, Tokyo, Japan). The number per cm² and volume of each focus were computed using the method of three-dimensional space quantitation from the data on two-dimensional planes as reported by Campbell et al. (15). For the application of this method, the diameter of each focus was calculated from the size of the intersections of the focus, regarding the outline of the focus as a circle. For example, the diameter of a focus with the size of ~0.002 mm² was calculated to be 50 µm.

Estimation of hepatic copper accumulation

The copper concentrations in the liver samples were measured with a flame atomic absorption spectrophotometer (Hitachi 208; Hitachi Ltd, Tokyo, Japan) as previously reported (16). In addition, accumulation of copper in liver tissues was demonstrated histochemically by the sulfide silver method (17).

Results

To examine the development of liver injury, plasma GOT, GPT and copper levels in each animal at death were measured as shown in Figure 1. The animals in group BD and group BD + DEN exhibited significantly high levels of plasma GOT, GPT and copper compared with those of the animals in group CuDD + DEN. Histochemical examinations of the liver tissues showed results consistent with measurements of copper content (Figure 1): almost no staining was observed in the hepatocytes in group CuDD + DEN animals, while those in the other groups showed strongly positive copper staining typical of the LEC rat liver (Figure 2). The high level of plasma copper is considered to be the result of the leakage of copper into blood from damaged hepatocytes that contain abnormal levels of copper. Chronological changes of these indicators of liver injury were measured in the LEC rats fed the BD or CuDD (Figure 1). At the age of 5 months the animals fed the BD showed a sudden increase in plasma GOT, GPT and copper levels, suggesting the onset of liver injury. However, no adverse findings were detected for those fed the CuDD. The abnormally high values were maintained in BD-fed rats compared with those fed the CuDD during the plasma monitoring after month 5, although the levels decreased in BD-fed animals after the highest values were recorded at 6 months of age.

Survival rates of the animals are shown in Figure 1. All the animals in group CuDD + DEN survived until being killed (nine animals survived /nine animals employed), while rats in groups BD and BD + DEN began to die from the age of 4 to 5 months. The survival rates of the animals in groups BD and BD + DEN were 43% (9/21) and 80% (16/20), respectively. The cause of death was considered to be by severe hepatitis resulting in liver failure because of the atrophy and dark-brownish changes of the livers and severe systemic jaundice found at autopsy.

The occurrence of chronic liver injury at the time of death in the rats in the groups BD and BD + DEN was proved by histomorphological examination. Degeneration and necrosis of hepatocytes with oval cell proliferation and malagocytic changes of hepatocytes characteristic of chronic liver injury of the LEC rat were observed in the liver sections from those animals. Putative preneoplastic lesions were occasionally identified morphologically in those livers. Mitotic figures were also detected frequently in the hepatocytes, not only in the preneoplastic lesions but also in the surrounding tissues (BD, BD + DEN). In contrast, the liver tissues from the rats in group CuDD + DEN showed an almost normal histological appearance, without any focal or nodular areas of preneoplastic or neoplastic lesions (Figure 3). Mitotic figures were rarely found (1.47/cm²). In this group no abnormalities suspected to be caused by the low copper level in the diet were detected either in the general condition or in autopsy

Fig. 1. Biochemical data on plasma and liver function and survival curves of the LEC rat. Plasma GOT, GPT, and copper increase with the decrease of survival rate (left column). *P < 0.05; **P < 0.01 (Student’s t-test) compared with CuDD + DEN.

Fig. 2. Copper staining of LEC rat livers (×40). Copper accumulation in hepatocytes is apparent in group BD + DEN (A), while almost negative staining was observed in hepatocytes in group CuDD + DEN (B).
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Fig. 3. Histopathology and GST-P staining of LEC rat livers. Each left and right column indicates H&E (×100) and GST-P staining (×40), respectively. Group BD (A and B) and group BD + DEN (C and D) show large foci with liver injury. Group CuDD + DEN (E and F) shows small foci without liver injury.

findings, GST-P staining of liver sections is also shown in Figure 3.

In order to evaluate an effect of chronic hepatitis resulting from copper accumulation on the promotion of preneoplastic lesions, the average volumes of GST-P-positive preneoplastic lesions were calculated from the data on two-dimensional planes. The results are summarized in Table I. These measurements provided evidence similar to that from two-dimensional data. In group BD there was a significantly small number of preneoplastic lesions compared with groups BD + DEN and CuDD + DEN, although preneoplastic lesions of large size were frequently observed in group BD. On the other hand, a great number of preneoplastic lesions appeared in the liver sections from the rats of both groups BD + DEN and CuDD + DEN. However, only GST-P-positive foci of small size were detected in group CuDD + DEN, whereas the sections from group BD + DEN included numerous large preneoplastic lesions (Figure 4). Furthermore, in group BD + DEN, preneoplastic lesions were significantly larger than in group BD. Namely, significantly large preneoplastic lesions appeared in the livers with chronic liver injury, i.e. in groups BD and BD + DEN, compared with the group without signs of chronic liver injury, group CuDD + DEN.

Even the development of HCC was observed in the liver of one animal in group BD + DEN. It was defined histologically as well-differentiated HCC with a trabecular structure. This lesion was excluded from the analyses of both the number and the area of GST-P-positive lesions because the liver section was almost totally occupied by the HCC.

Discussion

In experimental hepatocarcinogenesis, it is generally accepted that the size of GST-P-positive lesions implies the intensity of the promotion effect, while the number of the lesions in rat livers implies the intensity of the initiation effect. In the present experiments, the effects of the liver injury caused by copper accumulation on the process of hepatocarcinogenesis
of the LEC rat were examined by measuring the number and size of the lesions. The size of the lesions in the livers of rats fed the BD with DEN was significantly larger than that in the livers of rats fed the CuDD with DEN. Since the CuDD prevented LEC rats from developing liver injury due to an abnormal copper accumulation, these results clearly showed that chronic liver injury of the LEC rat promoted hepatocarcinogenesis.

There are several investigations supporting the concept of the participation of liver injury in hepatocarcinogenesis (9–11) because liver injury results in subsequent proliferation of hepatocytes, which presumably enhances the frequency of DNA damage and/or the fixation of DNA mutation (18–20). In the LEC rat, p-penicillamine (13) and trientine dihydrochloride (21) effectively inhibited not only liver injury but also subsequent development of HCC. On the other hand, endogenous growth stimuli such as HGF are reported in the LEC rat with chronic liver injury (22). In the present experiments, feeding the CuDD to the LEC rats prevented the development of both liver injury and preneoplastic lesions, presumably due to a marked reduction of hepatocytic degeneration and subsequent proliferation. These observations indicate that chronic liver injury caused by copper accumulation promoted hepatocarcinogenesis of the LEC rat, which necessarily required cell proliferation.

The effects of copper on the development of neoplasms are controversial. It is reported that copper plays an important role in DNA damage through generation of reactive oxygen species (23,24). Elevated levels of oxidative products of DNA, 8-hydroxydeoxyguanosine (25) and etheno–DNA adducts (26), were reported in the LEC rat liver with the accumulation of copper. On the other hand, copper has been reported to inhibit the incidence of chemically induced liver tumors (27,28). It is possible that there are at least two types of participation of copper in the LEC rat hepatocarcinogenesis. One is DNA damage resulting from the generation of free radicals taking part in the initiation and subsequent additional mutation of initiated hepatocytes. Multiple heritable changes in DNA caused by factors such as free radicals have been accepted as critical events for the carcinogenic process (18,29,30). In this way, copper is considered to participate in the development of liver cancer in the LEC rat.

The other is that copper accumulation may provide a selective growth environment for preneoplastic and neoplastic hepatocytes. We demonstrated that the majority of the preneoplastic liver lesions and HCCs show lower copper contents than the surrounding liver tissues (31), and that p21waf1/cip1/sdi-1, a negative regulator of the cell cycle, is expressed in normal tissues at a level much higher than in neoplastic lesions (32). Furthermore, the expression of p21waf1/cip1/sdi-1 in LEC rat livers is significantly reduced by CuDD, probably resulting in the recovery of growth activity of LEC rat hepatocytes (33). Thus, copper accumulation exerts a growth inhibitory effect on normal hepatocytes, on the one hand, and also stimulates secretion of HGF from non-parenchymal cells due to hepatocytic degeneration and death (22) on the other. Under these conditions, the cells, escaping from the effect of copper toxicity by lowering copper accumulation in the preneoplastic and neoplastic lesions could selectively proliferate in response to HGF from non-parenchymal cells and heparin-binding EGF in an autocrine fashion (34).

We have reported the high sensitivity of LEC rats to the hepatocarcinogen diethylnitrosamine (35). Recently, we found that the high susceptibility of LEC rats to hepatocarcinogens is genetically independent of abnormal copper accumulation in the liver (36), which may be quite different from human patients with Wilson’s disease. Thus, the LEC rat is also expected to be a useful animal model for investigations that clarify the relationship between chronic liver injury and the development of HCC.

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