0031-3998/92/3103-0253$03.00/0
PEDIATRIC RESEARCH

Vol. 31, No. 3, 1992
Printed in U.S.A.

Inherited Copper Toxicity in Long-Evans Cinnamon Rats Exhibiting Spontaneous Hepatitis: A Model of Wilson's Disease

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ABSTRACT. The copper concentrations in organs of developing Long-Evans Cinnamon (LEC) rats (2 d to 13 mo) were measured to elucidate the pathogenesis of their hereditary hepatitis. Hepatic copper contents of LEC rats were significantly higher than those of control rats (26 to 92 times higher). The subcellular distribution of hepatic copper indicated that the nuclear and large granular fractions had been saturated and the cytosol fraction contained about 70% of all the hepatic copper in LEC rats. The serum concentrations of copper and ceruloplasmin were significantly lower than those of control rats from the 4th wk (10–12% and 5–19%, respectively). Copper contents in kidney of LEC rats did not exhibit an increase over those of control rats until 12 wk, but then increased to nearly 40 times higher during fulminant hepatic failure. Accumulation of copper was not detected in the brain or small intestines of LEC rats until 13 mo. The hepatic copper concentration, its subcellular distribution, and serum copper concentration of F1 rats (LEC × Long-Evans Agouti) exhibited the same levels as those of Long-Evans Agouti rats. In addition to their similarity concerning inheritance of autosomal recessive means and clinical course, we found causality relating copper accumulation to the pathogenesis of the disease. We propose that LEC rats will be the most promising animal model for the study of Wilson's disease. (*Pediatr Res* 31: 253–257, 1992)

Abbreviations

LEC, Long-Evans Cinnamon
LEA, Long-Evans Agouti
AST, aspartate aminotransferase
Cr, creatinine
DW, dry weight

Spontaneous hepatitis associated with severe jaundice and anemia occurred in about 80% of an inbred strain of Long-Evans rats isolated from a closed colony at the center for Experimental Plants and Animals, Hokkaido University (1, 2). Two inbred strains were originally selected by coat color: LEC and LEA. Spontaneous jaundice was noticed for the first time in the offspring of the LEC rats at the 23rd generation of brother-sister mating, whereas none of the offspring of LEA rats developed the disease. Unique features of the disease were its late onset (16–23 wk), its close resemblance to fulminant hepatitis, and its high lethality (80%) within a week after onset of hepatitis. Furthermore, the surviving rats, which exhibited chronic hepatitis, spontaneously developed liver cancer with age (3). The genetic basis has been shown to be a single autosomal recessive mutation (4).

Wilson's disease is a form of serious hepatic, neurologic, or psychiatric disease, which was described in 1912 by Wilson (5) as "progressive lenticular degeneration: a familial nervous disease associated with cirrhosis of the liver." Existence of clinical subgroups among cases of Wilson's disease has long been postulated, which indicates that Wilson's disease is not homogeneous (6). Affected siblings in several inbred families who most likely inherited two copies of the same mutant allele have exhibited incongruous symptoms (7). Therefore, phenotypic variability in Wilson's disease may be due in part to an interaction of the Wilson's disease gene with a genetic or nongenetic modifier such as age. Hepatic onset occurred in 45% of the patients (8). In a few cases of patients with Wilson's disease, the course of hepatitis, accompanied by hemolytic anemia, may be so severe that the physicians almost invariably consider the illness to be an acquired episode of viral or toxic etiology. The primary pathogenetic mechanism in Wilson's disease is the metabolic defect, which is inherited by autosomal recessive means and is always associated with the gradual and progressive accumulation of copper in the liver (9). Autosomal recessive disorders of Bedlington terriers (10–14) and copper toxic milk mice (15, 16) have been studied as models of Wilson's disease. Excess hepatic copper has been found in the toad *Bufonidae marinus* (17) and the mute swan (18).

To elucidate the pathogenesis of spontaneous hepatitis in LEC rats, we paid particular attention to hereditary hepatic disease of autosomal recessive means in conjunction with late onset (19). Because of certain similarities of the disease exhibited in LEC rats to the hepatic type of Wilson's disease, we initiated studies of copper metabolism in LEC rats and discovered the inherited copper toxicity. In conclusion, we propose that LEC rats will be a new animal model for the study of Wilson's disease.

MATERIALS AND METHODS

Animals. Male and female LEC and LEA rats (Animal center of Hokkaido University, Sapporo, Japan) of uniform age were caged in groups of four to five and fed a basal diet (CE-2, Clea, Tokyo, Japan) *ad libitum.*

Longitudinal changes of serum and urine copper concentration. Six male and five female LEC rats and five male and five female LEA rats were housed in separate metabolic cages to take urine and blood samples every month after measuring body weight. The urine analyses were made using Combistix (Miles-Sankyou,

Received April 25, 1991; accepted October 10, 1991.
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Tokyo, Japan), and urine was stored frozen at −70°C for the measurement of copper and Cr concentration. Blood was taken from the cervical vein, and serum was separated immediately and stored frozen at −70°C for measurement of copper concentration. The copper concentration was measured by a flameless atomic absorption spectroscopy technique (graphite atomizer furnace), using an atomic absorption spectrophotometer (model 180-80; Hitachi, Hitachi, Japan). Urinary copper concentration was expressed as μmol/μmol Cr.

Development of copper content of organs. Several LEC and LEA rats aged 2 d, 1, 4, 8, 12, 16, 20, 24, 28, and 32 wk, and 13 mo were anesthetized by ether, and blood was taken by heart puncture. Saline-perfused liver, kidney, small intestine, and brain were taken and immediately frozen at −70°C. Parts of liver and kidney were fixed and used for pathologic studies. Serum was separated immediately and frozen at −70°C for the determination of ceruloplasmin, AST, and bilirubin.

Triplicate tissue samples of approximately 0.1 to 1.0 g were dried at 120°C for 20 h and digested in Aristar grade nitric, perchloric, and sulfuric acid (20:5:2), and copper contents were analyzed with a Hitachi atomic absorption spectrophotometer (model 180-80, Hitachi, Japan) using an air-acetylene flame.

Intracellular distribution of hepatic copper. The individual livers of female LEC (3-mo-old), LEA (3-mo-old), and F1 (LEC female × LEA male, 8-mo-old) rats were perfused with cold 0.25 M ultrapure sucrose and taken in order to be weighed. They were suspended in the same solution and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle. The procedure for subcellular fractionation was carried out as described by Evans *et al.* (20). The pellet, after being centrifuged for 10 min at 14 000 × *g*, containing both mitochondria and lysosomes, was referred to as the large-granule fraction. Each fraction was used for the purpose of copper analysis after digestion, as previously described. The recovery rate of copper in all four fractions in relation to the total homogenate was 95 ± 2.6% (SD).

Measurement of serum ceruloplasmin concentrations. Concentrations of ceruloplasmin were measured in triplicated samples of serum by the oxidase activity toward *p*-phenylenediamine (21).

Statistical analysis. Data are presented as the mean ± SD. For each assay, not less than three separate observations were made in independent experiments. The *t* test was used to compare the mean value of LEC rats with that of LEA rats.

RESULTS

Copper contents of the livers in developing LEC and LEA rats are shown in Figure 1. The copper concentration in LEC rat livers was significantly higher than that in LEA rats ($p < 0.025$–0.005). Copper contents of LEA rat livers were about 200.0 ± 6.7 and 198.6 ± 16.3 μg/g DW (these and subsequent values are for male and female rats, respectively) at 2 d and 209.7 ± 11.9 and 204.0 ± 16.7 μg/g DW at 1 wk. They then decreased to 12–16 μg/g DW and remained at that level until 32 wk. In contrast, copper contents of LEC rat livers were 597.8 ± 47.7 and 627.4 ± 74.8 μg/g DW at 2 d. After a decrease to 320.1 ± 56.5 and 380.0 ± 97.9 μg/g DW at 4 wk, copper gradually accumulated in the liver and reached the highest levels of 1153.3 ± 124.1 and 1201.7 ± 103.6 μg/g DW at 16 wk. Copper contents of liver in LEC rats that died from acute hepatitis were 574.9 ± 92.5 and 780.9 ± 121.7 μg/g DW, which were lower than those at 16 wk. After remission of acute hepatitis, the copper concentrations of surviving LEC rats' livers remained at 861.9 ± 23.0 to 631.1 ± 195.9 μg/g DW until 32 wk.

The hepatic copper concentration of F1 rats was the same as that of LEA rats (Female data is described in Table 1; that of male F1 rats was 15.2 ± 0.25 μg/g DW). This indicates that the copper accumulation in liver was inherited by autosomal recessive means. The serum copper concentrations were also the same as those of LEA rats (data not shown). In LEA and F1 rats, the

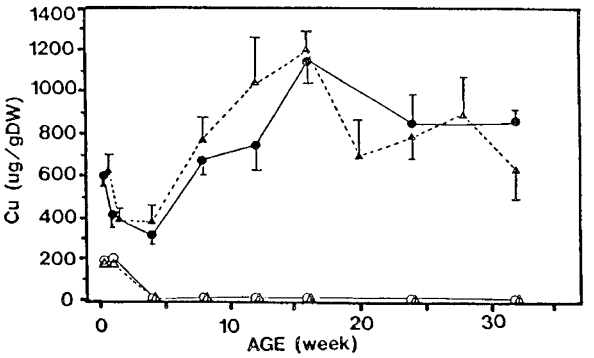

Fig. 1. Copper contents of liver in developing LEC and LEA rats. ●, LEC male; ○, LEA male; ▲, LEC female; △, LEA female. Copper contents of liver in LEC rats were significantly higher than those in age- and sex-matched LEA rats at any examined age ($p < 0.025$–0.005). The ratios of copper content of male and female LEC rats to LEA rats were 2 d: 3.0 and 3.2; 1 wk: 1.9 and 1.9; 4 wk: 26.3 and 30.2; 8 wk: 51.8 and 56.4; 12 wk: 53.1 and 87.5; 16 wk: 92.3 and 87.1; 24 wk: 62.2 and 48.2; and 32 wk: 63.3 and 47.0, respectively.

nuclear fractions of the liver contained the largest proportion of total hepatic copper (Table 1). Although this fraction of LEC rats contained over 20 times higher copper concentrations than those of LEA and F1 rats, the supernatant fraction contained about 70% of all the hepatic copper in LEC rats.

Copper contents of brain were 7.86–8.5 μg/g DW in LEC and LEA rats at 1 wk (data not shown). Until 12 wk, copper contents of LEC rat brains were lower than those of LEA rat brains (LEC/LEA ratios were 0.63–0.86, $p < 0.05$–0.005). After 16 wk, copper contents of male LEC rat brains were higher than those of LEA male rat brains (LEC/LEA ratios were 1.08 to 1.36, $p < 0.005$ at 32 wk). In contrast, copper contents of female LEC rat brains remained lower than those of LEA female rat brains until 24 wk (LEC/LEA ratios were 0.81 to 0.87, $p < 0.05$ at 16 wk), and overtook those of LEA rat brains at 32 wk (LEC/LEA ratio was 1.56, $p > 0.05$). Even after death caused by fulminant hepatitis, the copper contents of LEC rat brains were at the same levels as those at 16–20 wk. In general, copper contents of LEC rat brains were at their lowest at 12 wk and increased with age, reaching 16.3 ± 2.1 and 14.0 ± 0.1 μg/g DW at the age of 13 mo. Notably, no apparent differences were found between copper contents of LEC rat brains and those of LEA rat brains at 13 mo.

There was no significant difference between the copper concentrations of small intestines of LEC and LEA rats at any examined ages (data not shown). In both strains of rats, the copper contents were the highest at 1 wk. LEC rats that died because of fulminant hepatitis exhibited four to five times higher copper levels than LEC rats at 16 wk (data not shown).

The copper contents of the kidneys in developing LEC rats were lower than those of LEA rats until 8 wk ($p < 0.05$ at only 4 wk, Fig. 2). From 16 wk, copper levels of LEC rats appeared to be higher than those of LEA rats ($p < 0.05$ at 32 wk). On the other hand, those of jaundiced LEC female rats were 1600.7 ± 173.3 μg/g DW, which was 16 times higher than those of LEC female rats at 16 wk ($p < 0.005$).

Longitudinal changes of the serum copper concentrations of individual LEC and LEA rats are shown in Figure 3. On the average, the serum copper concentrations of male LEA rats were 13.6 ± 4.1 μmol/L at 4 wk and increased with age, reaching 24.9 ± 2.2 μmol/L at 24 wk (Fig. 3*C*). Those of female LEA rats were 14.1 ± 0.9 μmol/L at 4 wk and increased with age, reaching 28.2 ± 5.2 μmol/L at 24 wk (Fig. 3*D*). Serum copper concentrations of LEC rats were only 10 to 12% of those of LEA rats ($p < 0.05$–0.0005 with the exception of male rats at 20 wk, Fig. 3*A* and *B*), but those of jaundiced LEC rats increased over those of LEA rats (male, 55.1 ± 5.7 μmol/L; female, 47.0 ± 10.8 μmol/L). All female LEC rats expired from fulminant hepatitis before 24 wk.

Table 1. *Intracellular distribution of hepatic copper in LEC, LEA, and F1 rats**

Group (age)	Hepatic copper concentration†	N‡	LG	Mc	S
LEC (3 mo)	223.1 ± 6.8	13.7 ± 1.0	8.9 ± 1.1	4.6 ± 0.6	72.8 ± 1.1
LEA (3 mo)	3.7 ± 0.3	40.4 ± 6.1	20.9 ± 4.2	4.3 ± 0.5	34.4 ± 6.3
F1 (8 mo)	3.0 ± 0.5	34.7 ± 0.5	14.6 ± 3.6	7.2 ± 1.3	43.5 ± 4.1
LEC:LEA	$p < 0.001$	$p < 0.01$	$p < 0.02$	NS	$p < 0.01$
LEC:F1	$p < 0.001$	$p < 0.05$	NS	NS	$p < 0.05$
LEA:F1	NS	NS	NS	$p < 0.05$	NS

* N, nuclear; LG, large granular or mitochondrial and lysosomal; Mc, microsomal; and S, supernatant fraction.
† Expressed as mean ± SD in μg/g wet weight.
‡ Percentage of hepatic concentration in different fractions after calculations as μg/g wet weight (mean ± SD).

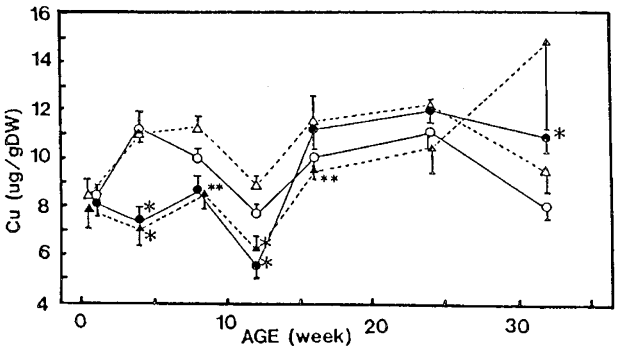

Fig. 2. Copper contents of kidney in developing LEC and LEA rats. ●, LEC male; ○, LEA male; ▲, LEC female; △, LEA female. *, $p < 0.005$ and **, $p < 0.05$ *vs* age- and sex-matched LEA rats.

Three male LEC rats survived, and their serum copper contents decreased to 5.0 ± 1.6 μmol/L at 24 wk. There was no evident correlation between serum copper concentrations and deaths from fulminant hepatitis.

Longitudinal changes in urinary copper concentrations of individual LEC and LEA rats are shown in Figure 4. On the average, LEA male rats excreted 2.78 ± 1.16 μmol of copper/μmol Cr in urine at 4 wk and 1.07 ± 0.50 μmol of copper/μmol Cr at 24 wk (Fig. 4*C*). LEA female rats excreted 2.80 ± 1.16 μmol of copper/μmol Cr at 4 wk and 3.76 ± 1.42 μmol of copper/μmol Cr at 24 wk (Fig. 4*D*). When urinary copper concentrations were expressed as μmol of copper/mL of urine, LEC rats appeared to excrete five to 12 times more copper in the urine than LEA rats. However, when urinary copper concentrations were expressed as μmol/μmol Cr, LEC rats excreted 2.8 to 3.8 times more copper in the urine, which was significantly higher only at 20 and 24 wk in males and at 12 wk in females ($p < 0.05$). Urinary copper excretion increased 3-fold in jaundiced LEC rats.

The serum ceruloplasmin concentration at 1 wk was 205.8 ± 45.7 and 192.2 ± 14.4 mg/L in male and female LEA rats, respectively (Fig. 5). It increased to 552.1 ± 84.6 and 589.0 ± 31.0 mg/L at 4 wk, respectively, and did not change significantly except for in male rats at 24 wk ($p < 0.025$). As can be seen, serum ceruloplasmin concentrations of LEC rats were only 5–19% of those of LEA rats after 4 wk ($p < 0.05$–0.0005). The ratio of copper to ceruloplasmin (μg/mg) was 2.4–2.7 in LEA rats, which indicated that one ceruloplasmin molecule contained nearly six copper atoms. The ratio was 4.7–6.9 in LEC rats, and it increased to 12.4 in jaundiced LEC rats, indicating an increase of nonceruloplasmin copper.

Table 2 shows the correlation of hepatic copper concentration to serum bilirubin concentrations, AST activities, and hepatic morphologic findings in LEC female rats. Two groups can be distinguished among 12-wk rats. Group A (12 wk), whose livers contained the same copper concentrations as those of 8-wk rats, exhibited normal serum bilirubin and AST values as well as normal morphologic findings. Group B (12 wk), whose livers

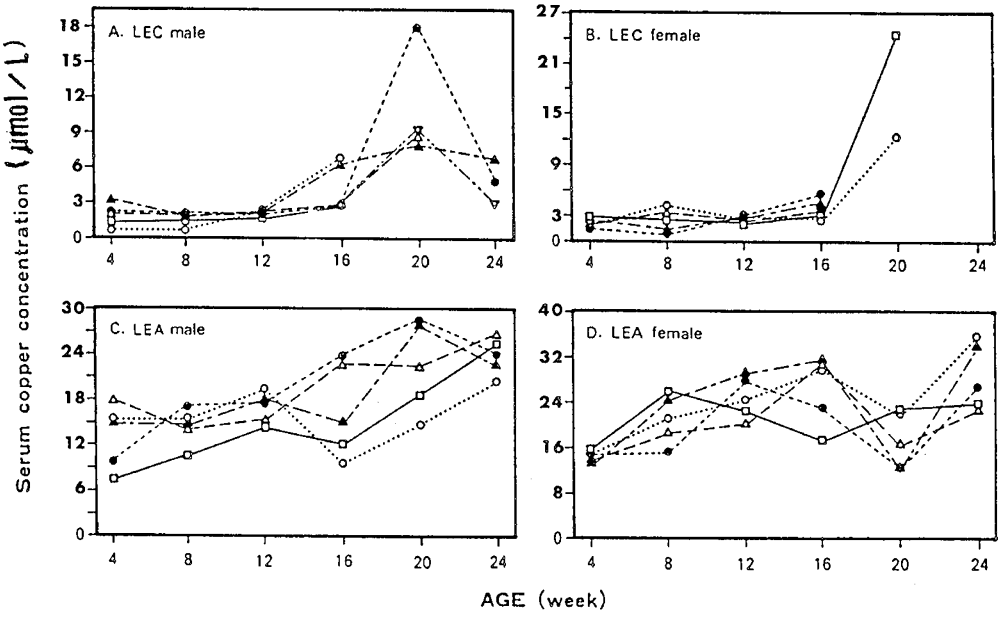

Fig. 3. Longitudinal change of serum copper concentrations in developing LEC and LEA rats. *Symbols* stand for individual rats of each group. LEC rats, six male and five female; LEA rats, five male and five female.

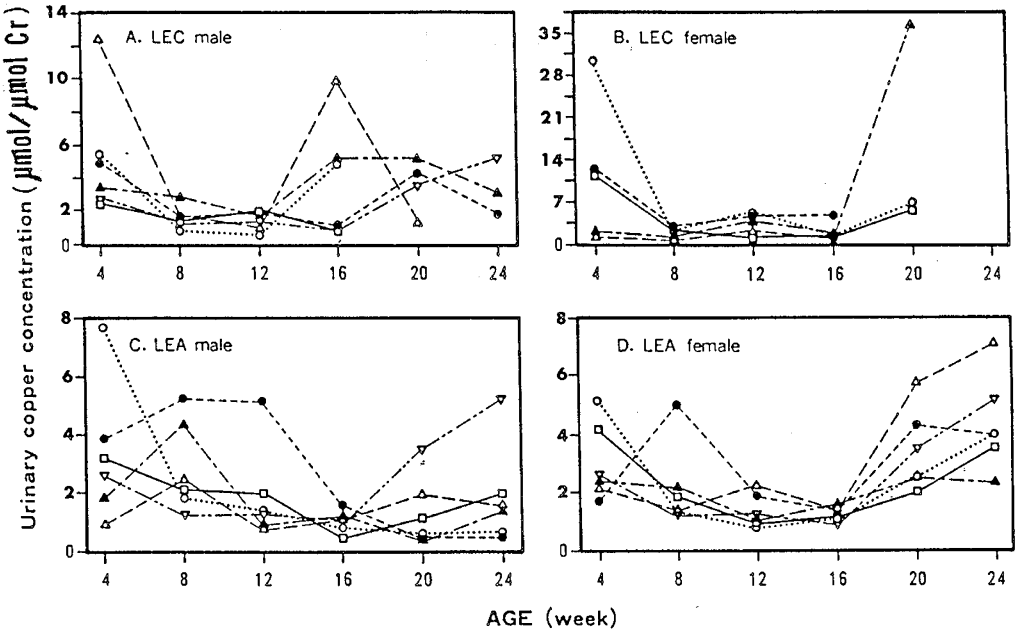

Fig. 4. Longitudinal change of urinary copper concentrations in developing LEC and LEA rats. *Symbols* are identical to those used in Figure 3.

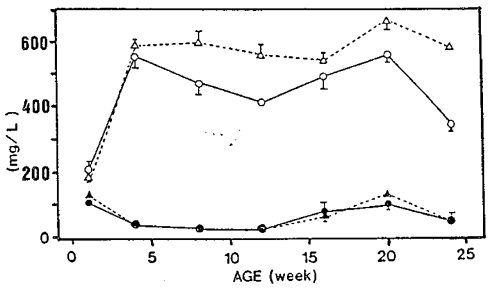

Fig. 5. Ceruloplasmin concentration of serum in developing LEC and LEA rats. ●, LEC male; ○, LEA male; ▲, LEC female; △, LEA female. $p < 0.005$ *vs* age- and sex-matched LEA rats.

contained significantly higher copper concentrations than group A, exhibited minor morphologic changes. Serum data revealed no jaundice, but rather an elevation of AST. Because there was an approximately 4-wk difference in the onsets of acute hepatitis (2), it is reasonable to assume that the copper contents and serologic data were not uniform in 12-wk LEC rats. At 20 wk, when severe morphologic changes (*e.g.* predominate necrosis with infiltration of a few inflammatory cells) are dominant, serum total bilirubin and AST were markedly elevated. Yet, copper concentrations of 20-wk rat livers were nearly identical to those in 8-wk rats, as if copper had been released from the liver because of necrosis of hepatocytes.

DISCUSSION

The discovery and study of animals with defects identical to those of human genetic disorders allows studies that are impossible to carry out on patients and may assist by furthering our understanding of the processes of copper metabolism. LEC rats bear a very close resemblance to those patients suffering from hepatic-type Wilson's disease accompanied by severe hepatic insufficiency and hemolytic anemia. In addition to demonstrating their similarities in the clinical course, we have demonstrated, by analysis of F1 rats, progressive copper accumulation in the liver that is inherited in recessive means. We have also provided the evidence that acute hepatic failure originates from copper toxicity.

Copper contents of liver in LEC rats were significantly higher than those in LEA rats at any examined age. When no histologic change was observed, the ratio of copper contents of LEC to LEA was about 30 at 4 wk and 50 at 8 wk. It increased to nearly 90 at 16 wk, shortly before the onset of fulminant hepatitis. These data indicate that the gradual and progressive copper accumulation in the liver had finally led to the onset of acute hepatitis. Copper content in the liver of LEC rats with jaundice was only 50 to 65% compared with 100% in those at 16 wk. This might suggest the release of copper from liver to blood and other organs, such as kidneys, which may have been the result of necrosis of hepatocytes. The predominant necrosis of liver was revealed by histologic examination (2).

Table 2. *Correlation of hepatic copper concentrations to serum bilirubin concentrations, AST activities, and hepatic morphologic findings in LEC female rats**

		Liver		Serum	
Age	*n*	Copper concentration (μg/g DW)	Morphologic findings	Total bilirubin (μmol/L)	AST (μkat/L)
12 wk A	3	725.7 ± 51.3	Normal	3.42 ± 0.17	3.93 ± 0.51
12 wk B	5	1209.4 ± 9.4†	Mild	3.76 ± 0.34	11.24 ± 0.58†
20 wk	5	694.4 ± 178.1	Severe	559.17 ± 107.73‡	16.99 ± 0.95†
28 wk	3	723.9 ± 121.9	Mild	3.42 ± 0.34	3.52 ± 0.97

* The values given are mean ± SD.
† Significantly different from 12 wk A, $p < 0.05$.
‡ Significantly different from all other values in the same column, $p < 0.005$.

The present findings concerning subcellular distribution of hepatic copper revealed that the cytosol fraction contained about 70% of all the hepatic copper in LEC rats, whereas the nuclear and large granular fractions contained nearly 80% of the hepatic copper in LEA rats. These findings substantiate the reports that nuclear and large granular fractions might protect hepatocytes from the toxic effect of the metal by its sequestration and that both fractions have apparent saturabilities (20, 22–23). Furthermore, these findings in LEC rats coincide with the intracellular distribution of hepatic copper in asymptomatic patients (24) as well as in one young patient (25) diagnosed as having Wilson's disease.

The mechanism of copper accumulation in LEC rats remains unknown. As in Wilson's disease, reduced excretion into bile and impaired incorporation into ceruloplasmin are suspected. Theories that have been advanced causally relating abnormalities of ceruloplasmin to the pathogenesis of Wilson's disease have not been proved true because of two types of individuals: patients with Wilson's disease who exhibit a normal concentration of ceruloplasmin and clinically unaffected heterozygous carriers of one Wilson's disease gene with a deficiency of ceruloplasmin (26). The concentrations of ceruloplasmin, measured by oxidation activities, and of serum copper in LEC rats were only 5–19% and 10–12%, respectively, of those of LEA rats. As for the ratio of serum copper to ceruloplasmin (μg/mg), LEC rats had 2- to 3-fold more nonceruloplasmin copper than LEA rats. LEC rats did not show a remarkable difference in concentration of serum immunoreactive ceruloplasmin before onset of hepatitis (Aoki T, personal communication). During jaundice, when copper and apoceruloplasmin might have been released from damaged hepatocytes, a nearly 5-fold increase of the ratio of serum copper to ceruloplasmin (μg/mg) was observed. These findings suggest that the incorporation of copper into ceruloplasmin might be impaired in LEC rats.

Because the biliary tract is the primary route for excretion of copper (27, 28), any defects in this system could lead to pathologic accumulation of copper. Reduced biliary excretion of copper in Wilson's disease has been observed (29–32). This has been attributed to defective lysosomes (32). When measured using radio-copper, absorption of copper has been found to be normal (33). Further examination of the mechanism of progressive accumulation of copper in liver and acute toxicity of copper after hepatic failure are under investigation in our laboratory. The treatment with chelating agents (triethylene tetramine) had a significant effect in protecting LEC rats from acute hepatitis (34).

Chronic progressive hepatic disease in Bedlington terriers is an inherited disease somewhat resembling Wilson's disease (11). Brain copper was reported to be normal (13) or often elevated (14), but cerebral effects were lacking. In LEC rats, a Kayser-Fleischer ring was not detected macroscopically until 13 mo after birth. Some LEC rats after 8 mo of age infrequently showed convulsions, whereas no LEA rats exhibited the symptom (19). Brain copper of 13-mo-old LEC rats was not significantly higher than that of age- and sex-matched LEA rats. Although there are certain differences between characteristic features of LEC rats and Wilson's disease, we propose that LEC rats will be the most promising animal model for the study of Wilson's disease.

Acknowledgments. The authors thank T. Mike and K. Ikuta for technical assistance.

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