RAPID COMMUNICATION Jpn. J. Cancer Res. 84, 219–222, March 1993

Abnormal Hepatic Iron Accumulation in LEC Rats

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The LEC (Long-Evans cinnamon) rat is a mutant strain displaying hereditary hepatitis and spontaneous hepatocellular carcinoma, and shows abnormal hepatic copper accumulation similar to that occurring in Wilson's disease. We evaluated the iron metabolism of LEC rats compared to LEA (Long-Evans agouti) rats. Hepatic iron and ferritin concentrations were remarkably increased depending on age in LEC rats but not in LEA rats. Increased hepatic iron is normally associated with decreased serum transferrin and total iron binding capacity in hepatic iron overload. In LEC rats, however, both serum transferrin and total iron binding capacity increased with increasing hepatic iron. This increase of serum transferrin and hepatic iron may be an additional important factor contributing to liver injury in LEC rats.

Key words: LEC rat — Iron metabolism — Ferritin — Transferrin

LEC[5] and LEA rats have been established from a closed colony of Long-Evans rats. LEC rats suffer from spontaneous hepatitis with jaundice developing around 4 months after birth, followed by death in 40% of rats due to fulminant hepatitis. The remaining rats recover, but exhibit chronic hepatitis and develop cholangiofibrosis or HCC.[1-3] In contrast, LEA rats develop no liver disease. Genetic analysis of LEC rats demonstrated that a single autosomal recessive gene is responsible for the hepatitis.[4] Recently, Li *et al.*[5,6] observed that LEC rats accumulate excess copper in the liver, but have decreased levels of serum copper and ceruloplasmin, a clinical presentation similar to Wilson's disease in humans.[7] The copper ions are usually present bound to metallothionein in the tissue and therefore are not toxic.[8] In the liver of LEC rats, however, metallothionein is saturated with copper, and excess free copper ions produce free radicals that result in liver damage.[9] However, the incidence of HCC in Wilson's disease is less than that seen in LEC rats.[10,11] Thus, HCC development may be due to more that just hepatic copper accumulation.

Iron similarly can induce free radical production.[9,12] We have therefore examined whether iron metabolism is abnormal in LEC rats. Our studies indicate that the serum level of transferrin increases abnormally, and excess iron as well as copper accumulates spontaneously in the liver of LEC rats.

Male LEC and LEA rats were maintained under conventional conditions at the Center for Experimental Plants and Animals of Hokkaido University. Wistar rats were obtained from Charles River Japan, Inc. The iron and copper concentrations in the liver were determined using an atomic absorption spectrophotometer with an air/acetylene flame (type 208; Hitachi). To produce anti-rat ferritin antibody, ferritin was purified from the liver of Wistar rats as previously described.[13] The purity of rat ferritin was 97% as evaluated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown). Anti-rat ferritin polyclonal antibody was produced by a standard immunization method using a New Zealand white rabbit and Freund's complete adjuvant. Concentrations of liver ferritin and serum transferrin were evaluated by Western blot analysis. Briefly, 0.2 g of the liver was homogenized with a Dounce homogenizer in 1 ml of PBS containing 1% SDS, and 1 m*M* PMSF. The homogenate was boiled for 2 min, then sonicated for 30 s and the supernatant was obtained by centrifugation at 12,000*g* for 10 min. Protein concentration was determined with a BCA protein assay kit (Pierce). Twenty micrograms of the liver extract or 1 μl of rat serum was electrophoresed on SDS-PAGE.[14] The separated proteins were then transferred electrophoretically onto Immobilon-P membranes (Millipore) by the method of Burnette.[15] The filter was incubated in PBS containing 10% nonfat dry milk for 1 h at room temperature, and incubated with 10 μg/ml of anti-rat transferrin antibody (The Binding Site Ltd.)

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[5] The abbreviations used are: LEC, Long-Evans cinnamon; LEA, Long-Evans agouti; HCC, hepatocellular carcinoma; SOD, superoxide dismutase; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TIBC, total iron binding capacity.

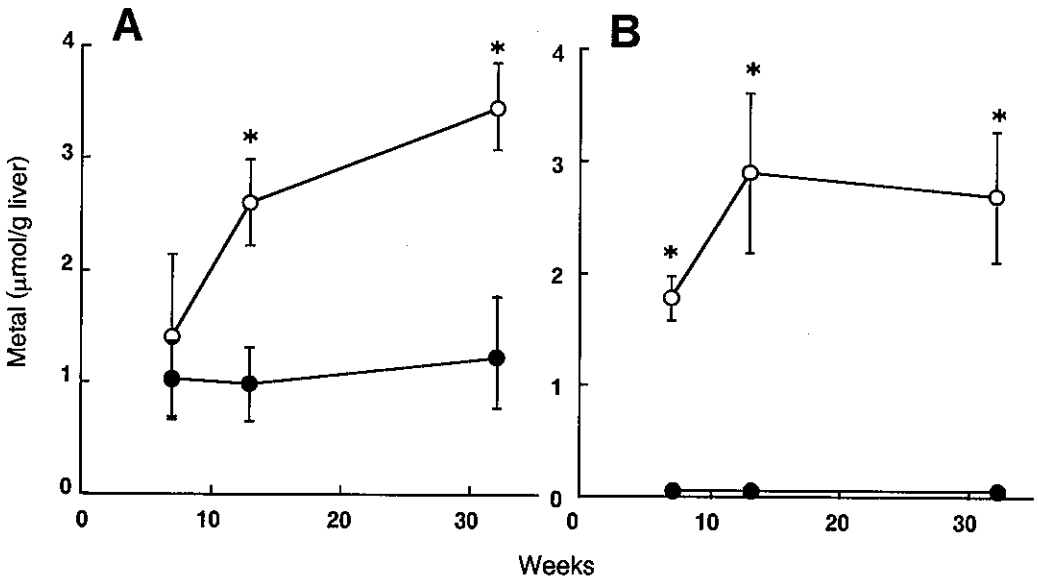

Fig. 1. Changes of hepatic iron (A) and copper (B) concentrations in LEC (○) rats and LEA (●) rats. The error bars express mean ± 1 standard deviation of 4 rats. *, $P < 0.001$ compared to LEA rats (Student's paired t test).

or anti-rat ferritin antibody (1:200) in PBS containing 0.05% Tween-20 for 1 h at room temperature. Antigen-antibody complexes were visualized by using an ABC kit (Vectastain). TIBC was measured by using TIBC micro-test Daiichi (Daiichi Radioisotope).

Hepatic iron and copper were determined as a function of age in LEC rats and, as a control, LEA rats. Although LEC rats had only slightly more hepatic iron than LEA rats at 7 weeks of age, hepatic iron rapidly increased thereafter in LEC rats to 3 μmol/g of liver, nearly three times that observed in LEA rats (Fig. 1). No increase in hepatic iron or copper was observed in LEA rats. To determine if the increased hepatic iron might induce synthesis of hepatic ferritin (as suggested by Drysdale and Munro),[13] liver homogenate was subjected to SDS-PAGE and Western blot analysis. As shown in Fig. 2, the anti-rat ferritin antibody reacted specifically with antigen from the liver of Wistar rats (lane 7), and ferritin expression clearly increased with age in LEC rats but not in LEA rats. The increased bands were evaluated by using densitometric analysis, and ferritin expression of 32-week-old LEC rats was found to be approximately 4-fold higher than that of 7-week-old LEC rats. We further examined the concentration of serum transferrin, because transferrin is usually decreased in iron overload. In contrast, however, this iron transport protein was increased approximately 2-fold in LEC rats compared to LEA rats (Fig. 3). TIBC, which reflects the iron-binding activity of serum transferrin, was comparably elevated in 7- to 32-week-old LEC rats relative to LEA rats (Fig. 4).

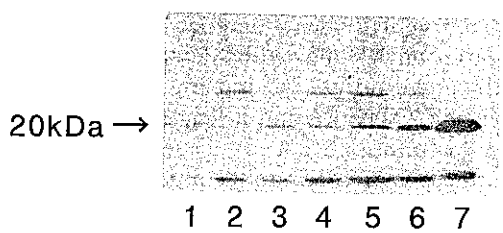

Fig. 2. Western blot analysis of liver ferritin from LEA rats (lanes 1–3) and LEC rats (lanes 4–6). Rats were 7-week-old (lanes 1, 4), 13-week-old (lanes 2, 5), and 32-week-old (lanes 3, 6). Lane 7, 100 ng purified ferritin from Wistar rats.

The LEC rats display abnormal copper metabolism and suffer spontaneous fulminant hepatitis and a high incidence of HCC. The excess copper accumulates in the liver of LEC rats, reaching its highest concentration just before the onset of hepatitis.[7] Togashi *et al.*[16] reported that D-penicillamine, a metal-chelating agent, could prevent the development of hepatitis in LEC rats, and suggested that copper ion accumulation in the liver is pathognomonic. Although copper accumulation in the liver is probably important in the pathogenesis of hepatitis, it is unclear why few patients with Wilson's disease develop HCC.[10] Perhaps LEC rats harbor additional abnormalities contributing to the development of hepatitis and cancer. Recent studies indicate that free metal ions can induce free radicals and lipid peroxidation reactions which may induce hepatic injury including hepa-

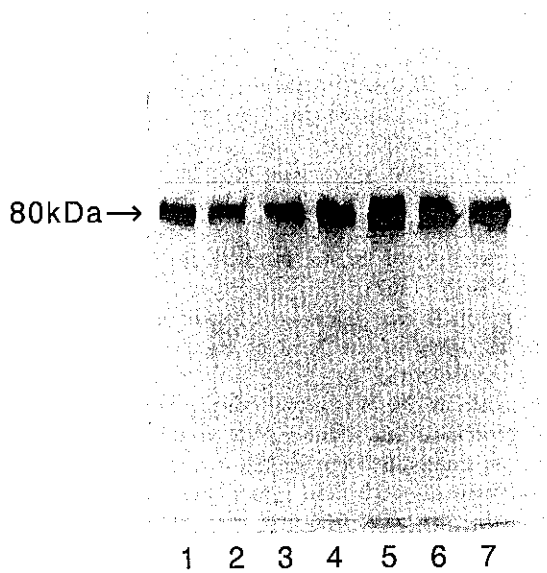

Fig. 3. Western blot analysis of serum transferrin of LEA rats (lanes 2–4) and LEC rats (lanes 5–7). Rats were 7-week-old (lanes 2, 5), 13-week-old (lanes 3, 6), and 32-week-old (lanes 4, 7). Lane 1, 1 μl of serum from a Wistar rat was loaded as a control.

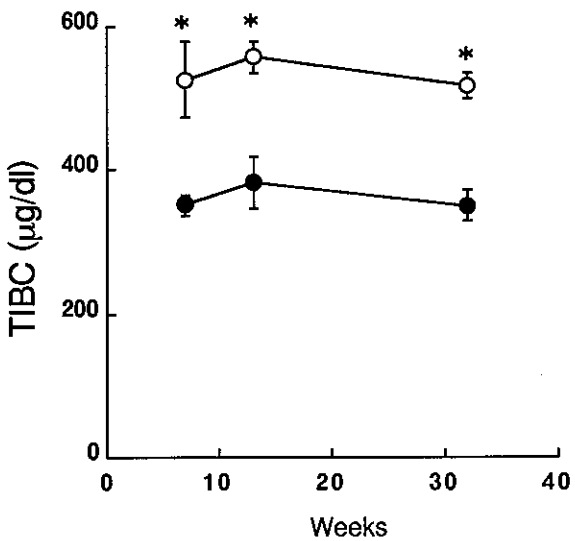

Fig. 4. TIBC in LEC (○) rats and LEA (●) rats. The error bars express mean±1 standard deviation of 4 rats. *, $P<0.001$ compared to LEA rats (Student's paired t test).

titis, liver cirrhosis, and HCC.[17,18] Metal ions such as copper, iron, manganese, and cobalt are known to be important for the production of free radicals, and iron is the most potent.[9]

We therefore examined iron metabolism in LEC rats. Hepatic iron in LEC rats was markedly increased by the age of 13 weeks, before onset of hepatitis. The hepatic iron concentration of LEC rats remained high in the period of chronic hepatitis at the age of 32 weeks, and was approximately 3-fold higher than in LEA rats. It is noteworthy that the increase in the molar concentration of iron is almost equal to that of copper in LEC rats, suggesting that iron may also have an important role in hepatic injury. Not unexpectedly, ferritin synthesis was enhanced in accordance with the hepatic iron accumulation in LEC rats. This result is consistent with the report that ferritin synthesis is induced by an increase in cytosolic iron and is regulated post-translationally by iron-responsive elements in the 5′-untranslated regions of ferritin mRNA.[19] On the other hand, serum transferrin in LEC rats behaved anomolously; it increased in LEC rats despite the increase of hepatic iron (Figs. 3 and 4). Normally, synthesis of serum transferrin is repressed by increases in hepatic iron. This is accomplished, in part, by two proteins that bind the promoter region of transferrin gene and respond to iron.[20] Our results suggest that the transcriptional regulation of hepatic transferrin synthesis may be impaired in LEC rats and the abnormal hepatic iron accumulation may be caused by the excess serum transferrin which carries iron from the reticulo-endothelial system and intestine to hepatic parenchymal cell. It is important to define the mechanism of iron accumulation in liver of LEC rats. One possibility is a decrease of life span of red blood cells (intravascular hemolysis by copper toxicity), as suggested in Wilson's disease in humans. In LEC rats, such a possibility cannot be ruled out completely. However, if hemolysis is increased and the resulting iron accumulates in the liver, serum transferrin and TIBC should be decreased. Neither is true for LEC rats, however. Perhaps in LEC rats, increased transferrin facilitates the intravascular iron transport from intestine and other tissues to iron storage sites such as the liver and spleen through transferrin receptor-dependent uptake of iron.

In conclusion, both iron and copper accumulation in the liver may be important for the development of liver damage in LEC rats. We are currently evaluating the effects of an iron-deficient diet on the onset of hepatitis and the development of HCC.

We wish to thank Dr. A. R. Frackelton, Jr. of Brown University, RI, USA for helpful discussions and a review of this manuscript. This work was supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

(Received November 2, 1992/Accepted January 12, 1993)

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