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A Marked Increase in Free Copper Levels in the Plasma and Liver of LEC Rats: an Animal Model for Wilson Disease and Liver Cancer

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Most of copper present in rat plasma and liver binds to caeruloplasmin and metallothionein, respectively, and is not redox active. However, free forms of copper including loosely bound forms to other molecules are redox active. We assessed the free copper in Long–Evans rats with a cinnamon-like coat color (LEC rats), an animal model of Wilson disease and liver cancer. Compared to those of control rats, the liver and plasma of LEC rats showed a marked elevation of free copper, especially at the stage of acute hepatitis, in parallel with an increase of total copper levels in the livers and a decrease of plasma caeruloplasmin (ferroxidase I) activity. At the onset of jaundice, the total copper levels, however, decreased in liver, but increased in plasma, while free copper levels in both liver and plasma remained higher. Free iron levels in both liver and plasma were also determined and did not change significantly, except for the case of plasma in jaundiced rats. The data are consistent with a proposal in which increased levels of redox active free copper in the liver of LEC rats catalyze Fenton-type reactions, producing a large flux of hydroxyl radicals that would play an important role in the observed liver dysfunction, leading to acute hepatitis, and, finally, hepatocarcinoma. This is the first demonstration that the free copper may participate in the pathophysiology of the LEC rats and Wilson disease.

Keywords: LEC rats, hepatitis, phenanthroline-detectable copper, bleomycin-detectable iron, reactive oxygen species, Fenton chemistry

Abbreviations: LEC rat, Long–Evans rat with cinnamon-like coat color; LEA rat, Long–Evans rat with agouti-like coat color; ROS, reactive oxygen species, EDTA, ethylenediamine tetra-acetic acid; TBA, thiobarbituric acid; PBS, phosphate-buffered saline; Cp, caeruloplasmin; *p*-APMSF, 4-amidinophenyl-methanesulfonyl fluoride hydrochloride; MT, metallothionein

INTRODUCTION

Wilson disease, an inherited disorder of copper metabolism, is characterized by hepatic copper accumulation, but low caeruloplasmin and copper levels in the plasma. Recent studies of

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the pathogenesis of Wilson disease indicate that it is due to a deficiency in a Cu-transporting ATPase.[1–3]

An inbred strain of Long–Evans rats with a cinnamon-like coat color (LEC rats), has been identified as a bona fide animal model of Wilson disease.[4–6] The LEC rat spontaneously and hereditarily develops acute hepatitis in about 4 months, and hepatoma at about 1 year after birth.[7] The clinical characteristics of the hepatitis resemble those of human fulminant hepatitis. The genetic analysis has revealed that a defect in the Cu transporting ATPase gene is responsible for the hepatitis, which is similar to Wilson disease.[8,9] It appears that LEC rats fail to incorporate Cu into caeruloplasmin in the Golgi apparatus.[10] Li *et al.*[5] reported Cu accumulation in several organs of LEC rats, especially the liver, during which serum levels of Cu and caeruloplasmin remained extremely low. They hypothesized that the cytotoxicity of excessively accumulated hepatic Cu is likely to cause necrotizing hepatic injury, leading to hepatitis in young rats and hepatomas in older ones. These features of the LEC rats are closely associated with Cu toxicity and, as a result, the disease can be considered a rodent form of Wilson disease.[11]

Evans *et al.*[12] reported that loosely bound Cu could not be detected in freshly prepared serum from patients with uncomplicated Wilson disease, whereas it was detectable in serum from a patient with fulminant hepatic failure. Ogihara *et al.*,[13] however, provided evidence that the plasma levels of loosely bound Cu were elevated in some patients with Wilson disease prior to the initiation of penicillamine therapy. As therapy proceeded, loosely bound Cu decreased to an undetectable level. The levels of free metals in tissues and plasma of LEC rats have not been precisely determined. Moreover, a molecular mechanism by which the decrease in caeruloplasmin activity associated with hepatic Cu accumulation, and their relationship to liver carcinogenesis in the LEC rat has not yet been established.

The present report describes attempts to detect free Cu and Fe in the plasma and liver of LEC rats using chelators phenanthroline and bleomycin and a comparison of these data with those of Long–Evans rats with an agouti-like coat color (LEA rats) as controls.

MATERIAL AND METHODS

Materials

DNA (herring testis), conalbumin (egg-white apotransferrin), and 1,10-phenanthroline were obtained from Sigma Chemical Co., St. Louis, USA. Standard solutions of Cu and Fe, benzamidine hydrochloride and ascorbate were purchased from Nacalai Tesque Co., Kyoto, Japan. Bleomycin sulfate was a gift from the Nippon Kayaku Co., Tokyo, Japan. 1-Butanol was purchased from Katayama Chemical Co., Tokyo, Japan. All other chemicals were of the highest grade available from Wako Pure Chemicals Co., Tokyo, Japan. All reagent solutions, and distilled water were passed through a Chelex column to remove transition metal ions before use.

Animals

Inbred strains of LEC rats, established by the Experimental Animal Laboratory of Hokkaido University, and LEA rats were bred under specific pathogen-free conditions at the Institute of Experimental Animal Sciences of Osaka University Medical School. Fifty-nine LEC rats and 74 LEA rats were sacrificed under anesthesia with diethyl ether and plasma and liver samples were collected. The LEC rats were classified according to age. The LEC rats under 12 (3–12) weeks old were arbitrarily classified as young. The LEC rats of 16–28 weeks old, 30–49 weeks old, and over 52 (52–127) weeks old, corresponded to acute hepatitis, chronic hepatitis and cancer stages, respectively. The pathological findings of organs in the respective ages of the LEC rats have been established earlier,[4,5,7] but in unclear cases, the

liver was carefully examined macroscopically or microscopically. The control LEA rats are classified in the same manner. We have designated the ages of the rats as young (< 12 W), young-adult (16–28 W), adult (30–49 W), and old (> 52 W).

Preparation of Plasma and of Tissue Homogenates

A 2–10 ml sample of blood was collected from each of 53 LEC rats and 80 LEA rats into lithium heparin. The resulting samples were centrifuged at 1,500 × *g* to separate the plasma, which was stored at 4°C within a day until required for phenanthroline-assay or stored under −30°C within a month until bleomycin-assay. Livers were obtained from 38 LEC and 24 LEA rats and were frozen directly in liquid nitrogen and stored at −80°C within 3 weeks prior to use. These storages give no eftects on the results of free Cu or Fe and ferroxidase activity as reported previously.[14] The preparation of liver tissue was carried out as described for other tissues.[15] In a pilot study, some livers were obtained after perfusion with phosphate-buffered saline (PBS) and their free Cu or Fe levels were compared with the group without perfusion. No major differences, however, were observed between these two groups (data not shown). Thus, in the following experiments, livers were simply excised without perfusion and washed with PBS prior to freezing. Adult LEC rats as well as those in the old stage had nodular lesions and hepatomas, respectively. The pathologically involved and non-involved tissues were carefully excised macroscopically and microscopically, and assayed separately. Samples obtained from 9 adult stage LEC rats and 12 old stage LEC rats were analyzed. Just before assay, the frozen liver was thawed, weighed, and homogenized on ice in specially cleaned glassware. The homogenizing buffer was 0.17 M Tris-HCl pH 7.4 containing 5 mM benzamidine hydrochloride and 10 μM 4-amidinophenylmethanesulfonyl fluoride hydrochloride (*p*-APMSF), from which contaminating metal ions had been removed by prolonged dialysis against conalbumin. Homogenates were centrifuged at 1,500 × *g*, 4°C for 1 h and the supernatant was ultra-centrifuged for 1 h at 354,000 × *g*. The final supernatant was loaded into an Ultrafree-MC 5,000 (Millipore Corp., Bedford, USA) ultrafiltration cell and centrifuged at 4°C for 30 min at 5,000 × *g*. The clean filtrate (approximately 0.5 ml) was used immediately for Cu or Fe determination. Protein concentrations in the samples were measured by the BCA method (Pierce).

Measurement of Total Copper and Total Iron Concentrations

The total Cu and the total Fe concentration of plasma and liver were measured by atomic absorption spectrometry using a Z-8000 Polarized Zeeman Atomic Absorption Spectrophotometer (Hitachi, Tokyo, Japan). The samples were diluted 10–50-fold using the same buffer. An aliquot of each sample, and a standard solution (10 μl), was directly loaded into a cuvette, and measurements were performed at 324.8 nm for Cu and 248.3 nm for Fe.

Phenanthroline Assay for Free Copper

This was carried out as previously described.[16] The 1,10-phenanthroline (1.98 mg) was first dissolved in 0.2 ml of ethanol and then made up to 10 ml of water to give a final concentration of 1 mM. DNA was prepared by allowing a 1 mg/ml solution to stand overnight at 4°C with a sealed dialysis tube containing Chelex resin inserted in the solution. The 2-mercaptoethanol solution was prepared by adding 0.4 ml to 100 ml of water. All reagents were sufficiently stable when stored at 4°C. The reaction mixture was made by adding the following reagents in the order stated: 0.4 ml of herring testis DNA (1 mg/ml). 0.1 ml of 1,10-phenanthroline (1 mM), 50 μl of NaN_3 (100 mM), 50 μl of Cu standard or sample, 0.2 ml of phosphate buffer, pH 6.5 (0.1 M), and 0.1 ml of

2-mercaptoethanol (0.4% v/v). New disposable plastic tubes and pipette tips were used throughout. Each sample had its own blank prepared as described above with the omission of the 1,10-phenanthroline solution but with the addition of 0.1 ml of water. A standard curve for Cu concentrations ranging from 1 to 10 μM was established by using $CuCl_2$. Tubes were vortexed after addition of each reagent, then incubated at 37°C for 1 h in a shaking water bath. Then 0.1 ml of 0.1 M EDTA was added to stop the reaction, followed by 0.5 ml of 1% (w/v) TBA in 50 mM NaOH and 0.5 ml of 28% (w/v) trichloroacetic acid. The contents were transferred to glass tubes and then heated at 100°C for 5–8 min. After cooling, the resulting pink chromogen was extracted into 3.0 ml of 1-butanol by vortex-mixing. After centrifugation at 1,500 × *g* for 10 min, the fluorescence of the 1-butanol phase was read at 553 nm with excitation at 532 nm.

Bleomycin Assay for Free Iron

This was carried out as previously described.[17–19] Bleomycin sulfate was dissolved in distilled water to give a stock solution of 1.5 units/ml. Ferric chloride used for Fe standards in the bleomycin assay was dissolved in acidic water to avoid precipitation of Fe from solution at neutral to alkaline pH values, when Chelex-treated-water was used (see Materials). Contaminating Fe was removed from the 1 M Tris-HCl buffer pH 7.4 by placing sealed dialysis tubing containing 5% conalbumin and bicarbonate in the solution. The buffer was left for 48 h at 4°C before use, and during this time the conalbumin became pink as Fe was bound to it.

A 0.4 ml of DNA (1 mg/ml was prepared in a similar manner to that used in the phenanthroline-Cu assay), 20 μl of bleomycin sulfate (1.5 units/ml), 0.1 ml $MgCl_2$ (50 mM), 20 μl of sample or Fe standard, 0.1 ml of 1 M Tris-HCl buffer pH 7.4, and 50 μl of newly prepared 7.5 mM L(+)-ascorbic acid were added to new clean plastic tubes and incubated at 37°C for 1 h. After incubation, 0.5 ml of TBA (1% w/v in 50 mM NaOH) and 0.5 ml of HCl (25% v/v) were added to each tube, which was then heated at 100°C for 5 min. After color development, the tubes were cooled and the resulting pink chromogen was extracted into 1.5 ml of 1-butanol. The tubes were centrifuged at 1,500 × *g* for 10 min to separate the phases and the absorbance of the clear upper organic phase containing the chromogen was measured at 532 nm.

Ferroxidase Activities

Total ferroxidase activity was assayed by measuring the oxidation of ferrous ions to the ferric state, at pH 6.5, which bind to apotransferrin to produce a pink complex (A_{460} nm).[20] In this assay, conalbumin (egg-white apotransferrin) was substituted for apotransferrin. The specific contribution by caeruloplasmin (ferroxidase I)[21] was evaluated as that inhibitable by 1 mM azide. Ferroxidase II activity is also found in stored human plasma samples,[14] and is due to an oxidized lipid–Cu-protein complex which has enzyme-like activity but is not inhibited by azide.

Statistical Analyses

All statistical analyses were performed with StatView 4.5 from Abacus, Berkeley, CA. The statistical significance of values between groups was evaluated with non-parametric Mann–Whitney *U*-test. Paired values between nodules or hepatoma lesions and non-involved tissue extracts from the same rat were evaluated with the Wilcoxon test. $p < 0.05$ was considered as significant.

RESULTS

Copper Levels in Plasma

Levels of total and free Cu were determined in the plasma of rats as a function of age. As shown in Figure 1A, the total Cu levels in the plasma of

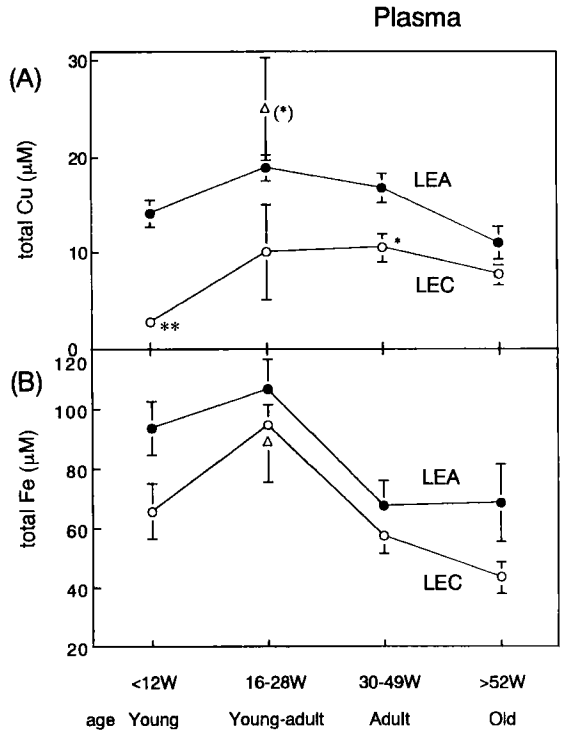

FIGURE 1 Total copper (A) and iron (B) levels in plasma of LEC and LEA rats. The young-adult age (16–28 W) LEC rats is divided into two groups without (J−) or with (J+) jaundice. The closed and open circles (●,○) and open triangle (△), correspond to the LEA, LEC including J−, and LEC including J+, respectively. The values are means ± SE; $n = 4$ to 28 for each group. The p-values using Mann–Whitney U-test are represented as $^{*}p < 0.05$; $^{**}p < 0.0005$ vs LEA rats. These symbols in parenthesis accompanying △ are vs the LEC J− rat using student t-test.

LEC rats at all ages were lower than those of the LEA rats, except for young-adult LEC rats with jaundice, as was reported previously.[5,11] As shown in Table I, the free Cu of young-adult LEC rats with or without jaundice was, however, significantly higher than that of the age matched control LEA rats ($p < 0.05$). In the plasma of young and young-adult LEC rats without jaundice, the proportions of free Cu to the total Cu were approximately 1:5, and were extremely higher than those in the age matched control LEA rats. After 30 weeks of age, the LEC rats showed significantly lower levels of free Cu in their plasma than the age matched control LEA rats ($p < 0.05$).

Copper Levels in Liver

We also quantified both total and free Cu levels in extracts of LEC and LEA rat livers. The LEC rats at the chronic hepatitis and cancer stages had nodular lesions and hepatoma, respectively. The analyses were carried out separately for normal (non-involved; N) tissues and nodule or hepatoma (H) lesions. The levels of total Cu in LEC rats were much higher than those in the control LEA rats for all ages, as shown in Figure 2A. These levels decreased in the LEC rats with jaundice. As shown in Table II, the free Cu levels of LEC rats were also higher than those of the age matched control LEA rats ($p < 0.05$ under young-adult age). This was particularly so for LEC rats at the young-adult age, when the rats develop fulminant hepatitis, in that some of the free Cu levels were markedly increased ($\sim 15\,\mu$mol/g-protein) in parallel with an increase in the total Cu in the liver. The proportion of free Cu to the total Cu was also very high, and approximate mean values exceeded 30%. Although levels of total Cu in the nodules or hepatoma lesions (H) were significantly lower than the surrounding non-involved tissues (N), the free Cu levels did not differ greatly.

Iron Levels in Plasma

Figure 1B and Table I show the levels of total and free Fe in the plasma of rats, respectively, as a function of age. Total Fe levels in LEC rat plasma were slightly lower than those in LEA rats at all ages. The free Fe levels in LEC rat plasma are not very different from those of LEA rats, except for those in the LEC rats with jaundice. The free Fe of the LEC rats with jaundice, however, was significantly higher than those of the age matched control LEA rats ($p < 0.005$) and the young-adult LEC rats without jaundice ($p < 0.05$). The proportion of free Fe is also nearly the same between the LEC and LEA rats, except for the LEC rats with jaundice (Table I).

TABLE I Free copper or iron levels in plasma of rats

Weeks age	Plasma free copper (μM)		Plasma free iron (μM)		The stage of LEC rats
	LEA	LEC	LEA	LEC	
< 12 W young	1.13 ± 0.29 (7.4 ± 3.0%)	0.71 ± 0.31 (21.8 ± 9.5%)	1.51 ± 0.33 (1.6 ± 0.3%)	3.20 ± 1.05 (2.8 ± 0.9%)	Young
16–28 W young-adult	0.76 ± 0.19 (5.2 ± 1.4%)	2.87 ± 0.94* (20.2 ± 9.6%) J+ 2.13 ± 0.61* (6.6 ± 1.9%)	1.66 ± 0.38 (1.3 ± 0.3%)	1.69 ± 1.34 (2.4 ± 1.9%) J+ 6.83 ± 3.78**(*) (8.5 ± 4.6%)***	Acute hepatitis
30–49 W adult	1.07 ± 0.21 (11.0 ± 2.7%)	0.30 ± 0.13* (7.5 ± 3.9%)	0.40 ± 0.09 (0.6 ± 0.2%)	0.56 ± 0.23 (0.8 ± 0.2%)	Chronic hepatitis
> 52 W old	1.14 ± 0.29 (8.6 ± 2.1%)	0.26 ± 0.14* (4.1 ± 1.7%)	0.51 ± 0.21 (0.7 ± 0.3%)	0.25 ± 0.08 (0.6 ± 0.2%)	Cancer

The units of free ion level are expressed as μM. Values are means ± SE; $n = 4$ to 27 for each group. Numbers in parentheses are the percent of free metal levels in each sample relative to the total. The young-adult age (16–28 W) of LEC rats is divided to two groups with (J+) or without jaundice. *P*-values are evaluated for LEC vs LEA rats with Mann–Whitney *U*-test. $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$. These symbols in parentheses are evaluated *p*-values between LEC rats with (J+) and without jaundice.

Iron Levels in Liver

Figure 2B and Table II show the levels of total and free Fe in the liver of rats, respectively, as a function of age. In contrast to Cu, all levels of total and free Fe and the proportion of free Fe in LEC rat livers were lower than those in LEA rat livers, except for hepatoma lesions (H) of LEC rats at old age. Free Fe was further decreased in LEC rat livers with jaundice, although it was not statistically significant. The nodules or hepatoma lesions (H) had higher levels of total and free Fe and a higher proportion of free Fe than the non-involved tissues (N).

Ferroxidase Activities in Plasma

Figure 3 shows changes in the plasma ferroxidase activities of LEC and LEA rats during aging. The LEC rats had lower total ferroxidase activities than LEA rats at all ages. The lack of ferroxidase I activity in LEC rats contributed to the lower total activities, compared to LEA rats at all ages. The ferroxidase I activity, which corresponds to the caeruloplasmin dependent oxidase activity, as well as native caeruloplasmin levels were lower for all ages, as previously reported,[11] but markedly increased for both the young and young-adult ages. Ferroxidase I activity in the

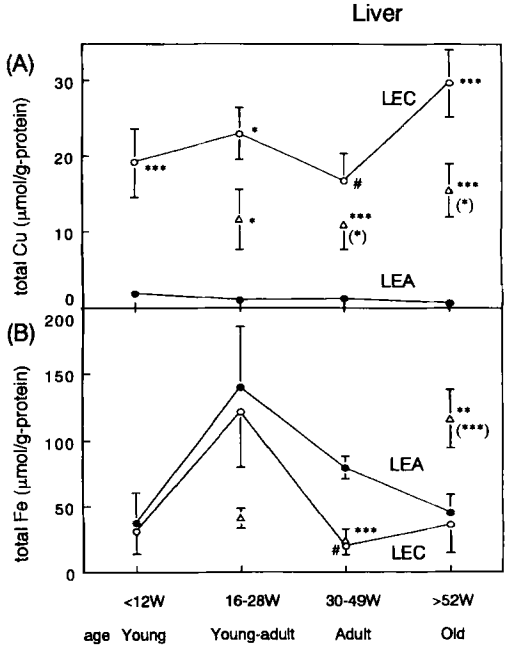

FIGURE 2 Total copper (A) and iron (B) levels in livers of LEC and LEA rats. The young-adult age (16–28 W) of LEC rats is divided into two groups without (J−) or with (J+) jaundice. The livers excised from the adult (30–49 W) and old (> 52 W) age LEC rats are divided into two portions, non-involved tissues (N) and those containing nodules or hepatoma lesions (H). The closed and open circles (●,○) and open triangle(△), correspond to LEA, LEC including J− or N, and LEC including J+ or H, respectively. The values are means ± SE; $n = 4$ to 12 in each group. The *p*-values using Mann–Whitney *U*-test are represented as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$; $^{\#}p < 0.001$ vs LEA rats. These symbols in parenthesis accompanying △ are vs N using Wilcoxon test.

TABLE II Free copper or iron levels in liver of rats

Weeks age	Liver free copper (μmol/g-protein)		Liver free iron (μmol/g-protein)		The stage of LEC rats
	LEA	LEC	LEA	LEC	
< 12 W Young	0.03 ± 0.03 (2.5 ± 2.2%)	0.21 ± 0.06 * (1.7 ± 0.6%)	0.19 ± 0.11 (0.6 ± 0.1%)	0.05 ± 0.02* (0.1 ± 0.0%) ***	Young
16–28 W Young-adult	0.01 ± 0.004 (1.9 ± 0.9%)	5.1 ± 4.8* (31.1 ± 29.8%) J + 5.2 ± 4.2 * (30.1 ± 15.8%) *	1.1 ± 0.4 (0.9 ± 0.4%)	0.64 ± 0.46 (0.4 ± 0.2%) J + 0.30 ± 0.09 (0.8 ± 0.3%)	Acute hepatitis
30–49 W Adult	0.05 ± 0.01 (7.4 ± 2.4%)	N 0.13 ± 0.06 (0.6 ± 0.3%) * H 0.13 ± 0.06 (1.1 ± 0.3%) *	0.81 ± 0.15 (1.0 ± 0.1%)	N 0.04 ± 0.02† (0.5 ± 0.1%) ** H 0.10 ± 0.02 ***(*) (0.8 ± 0.2%) (*)	Chronic hepatitis
> 52 W Old	0.03 ± 0.01 (4.3 ± 1.5%)	N 0.14 ± 0.05 (0.7 ± 0.3%) *** H 0.29 ± 0.13 * (1.8 ± 0.7%) *(**)	0.17 ± 0.06 (0.2 ± 0.1%)	N 0.11 ± 0.04 (0.2 ± 0.1%) H 0.45 ± 0.17 (**) (0.6 ± 0.2%)	 Cancer

The units of free ion level are expressed as μmol/g-protein. Values are means ± SE; $n = 4$ to 12 for each group. Numbers in parentheses are the percent of free metal levels in each sample relative to the total. The young adult period (16–28 W) of LEC rats divided to two groups with (J +) or without jaundice. In LEC rats over 30 W, metal levels were determined separately for non-involved tissues (N) and nodules or hepatoma lesions (H). *P*-values are evaluated for LEC vs LEA rats with Mann–Whitney *U*-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; †$p < 0.001$. These symbols in parenthesis are evaluated *p*-values between the H and N liver tissues with Wilcoxon test.

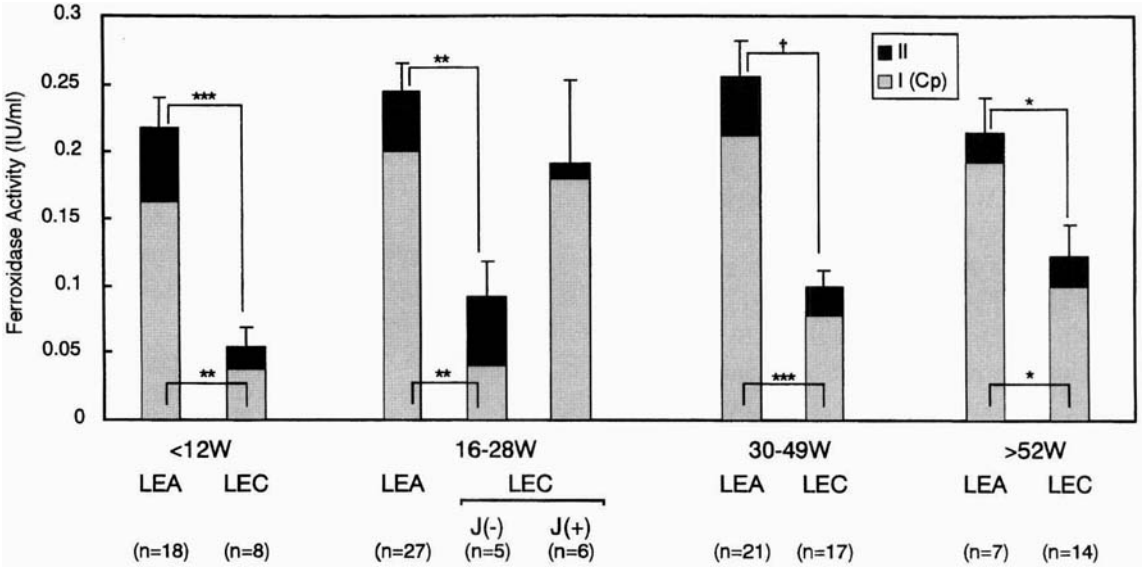

FIGURE 3 Ferroxidase activities in plasma of LEC and LEA rats. The gray and black columns correspond to ferroxidase I (caeruloplasmin; Cp) and ferroxidase II activities, respectively. The values are means + SE; *n* is noted under each column. *P*-values were evaluated for I(Cp) and total (I + II) activities of LEA vs LEC rats using the Mann–Whitney *U*-test. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; † $p < 0.0001$. The acute hepatitis stage (16–28 W) of LEC rats is divided into two groups without (J−) or with (J +) jaundice.

plasma of LEC rats with jaundice was not as low as the other groups.

DISCUSSION

In this study, we tried to understand participation of Cu and Fe in liver failure and hepatocarcinogenesis. The levels of total Cu in LEC rat livers were significantly higher than those in LEA rat livers during aging (Table II), as previously reported.[5,11] The majority of Cu in the liver of LEC rats is bound to MT.[22–28] The levels of Cu-MT in the LEC rat liver exceed 2 mg/g liver, which is 80–150-fold higher than basal levels of normal rats.[23,26] We also showed for the first time that the free Cu level in the LEC liver at young-adult age was about 500-fold higher than that in the LEA liver and much higher even than in the LEC liver at the other ages. The LEC rats with jaundice have higher plasma total Cu levels and lower liver total Cu levels than those without jaundice, as previously reported.[27]

As transition metal ions have variable valencies, Cu and Fe can readily transfer electrons to molecular oxygen to form reactive oxygen species (ROS).[29] For this reason the body normally keeps Cu and Fe in safely sequestered forms which limit their ability to take part in free radical reactions.[30] When Cu or Fe ions are released from sequestration and become free forms, they have the ability to catalyze the formation of aggressive and damaging species such as hydroxyl radical (•OH). Thus, Cu and Fe ions can form •OH by superoxide-driven Fenton chemistry, in which superoxide acts as a metal ion reductant, and as a source of hydrogen peroxide, as shown below:

$$[1]\quad O_2^{\bullet -} + Cu^{2+}(Fe^{3+}) \rightarrow Cu^{+}(Fe^{2+}) + O_2$$

$$[2]\quad H_2O_2 + Cu^{+}(Fe^{2+}) \rightarrow Cu^{2+}(Fe^{3+}) + OH^{-} + {}^{\bullet}OH$$

$$H_2O_2 + O_2^{\bullet -} \rightarrow OH^{-} + {}^{\bullet}OH + O_2$$

•OH is generally thought to play important roles in the genesis of inflammation and carcinogenesis.[30,31] It is likely that such free forms of Cu and Fe are loosely bound to other molecules such as histidine, citrate, acetate, phosphate, and proteins in the plasma.[32]

Thus the following scheme may be proposed for free Cu in hepatic failure and liver carcinogenesis. Cu bound to MT would keep accumulating in accord with induction of MT in the liver. Free Cu starts increasing when its amount is beyond capacity of MT at young-adult age. Because free Cu is highly toxic by catalyzing the Fenton reaction, severe liver failure corresponding to acute hepatitis would be caused. In the presence of hydrogen peroxide, production of •OH was actually demonstrated by ESR for Cu containing MT purified from LEC rat.[25] The amount of •OH was proportional to the amount of cuprous ions liberated from MT. Levels of lipid peroxidation whose formation is enhanced by •OH were significantly higher in symptomatic LEC rats at 4 months of age than in those of age-matched asymptomatic LEC and normal rats.[33]

Lower native caeruloplasmin levels as judged by ferroxidase I activity would be a cause of high free Cu levels in the LEC rat plasma (Table I and Figure 3). On the contrary, normal caeruloplasmin activity in the LEC rat with jaundice accounted for decrease in the free Cu fraction in its plasma. During the adult (chronic hepatitis) age, liver is regenerated and new hepatocytes can synthesize caeruloplasmin to some extent, thereby decreasing the levels of free Cu in the liver and plasma of LEC rats.

Free Fe as detected by the bleomycin assay is not present in the plasma of normal healthy adult humans.[18] It was, however, present in trace amounts in the plasma of some of the LEA control rats and the LEC rats, with the highest levels appearing in the acute hepatitis stage group of the LEC rats with jaundice (Table I). Trace amounts were also detected, but not at high levels, in liver extracts (Table II). The LEC rats with jaundice, however, showed significantly higher levels of

free Fe in the plasma but not in the liver. The total Fe levels in the livers of LEC rats without jaundice are slightly higher than those of the LEC rats with jaundice (Figure 2B). This suggests that, in the acute hepatitis age, the accumulated Fe in the liver, which exists as the bound form, is released from destroyed hepatic cells to the plasma as the free form. Thus, free Fe level in liver with jaundice was eventually lower even though free Fe level in liver of rats without jaundice was higher. Hemolysis and hemoglobin destruction occurs as a result of the Fenton reaction with the release of free Cu and free Fe. In addition, capacity of Fe metabolism in liver is destructed due to fluminant hepatitis,[34] resulting in an increase in the level of free Fe in plasma. Kato *et al.*[35] indicated that an Fe-deficient diet repressed the total Fe in liver or serum after 10 W and that non-heme or free Fe in the liver at 15 W and prevented fulminant hepatitis in young-adult LEC rats. On the regular diet employed in this study, the total Fe level in the liver of LEC rats initially decreases after the acute hepatitis stage, as previously reported,[29] but then increases in cancerous lesion at old (cancer) age. Free Fe levels in the liver of LEC rats change in nearly the same manner according to age. This suggests that after a long period, Fe metabolism may be impaired. In particular, cancerous hepatic cells may lack normal Fe metabolism and have much higher total and free Fe levels.

Extensive oxidative reactions following the release of over-accumulated tissue Cu, including DNA damage by $^{\bullet}OH$, would well occur at the acute hepatitis stage, eventually leading to hepatocarcinogenesis at a later age.[36] We have, for the first time, been able to relate free Cu and Fe to the characteristic phases of tissue damage observed in LEC rats, which provides a unique model for hepatocarcinogenesis.

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