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Hepatic Copper Accumulation Induces DNA Strand Breaks in the Liver Cells of Long-Evans Cinnamon Strain Rats

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Effects of accumulation of copper and iron on the production of DNA strand breaks were investigated in Long-Evans Cinnamon (LEC) strain rats that spontaneously develop fulminant hepatitis. Copper and iron accumulated in the liver of LEC rats in an age-dependent manner from 4 to 15 weeks. Low-copper food prevented the accumulation of copper in the liver, but did not prevent accumulation of iron. When the amounts of DNA single strand breaks were estimated by comet assay, the number of DNA strand breaks in the liver cells of rats fed standard food increased with age from 4 to 15 weeks. The number of DNA strand breaks in the liver cells from rats fed low-copper food were the same as those of rats at 4 weeks of age. Thus, the copper accumulation in the liver of LEC rats induced DNA strand breaks, but accumulation of iron did not. © 2000 Academic Press

Key Words: **comet assay; copper; DNA strand breaks; LEC rats; liver cells.**

An inbred strain of Long-Evans Cinnamon (LEC) rats was established as a mutant strain that spontaneously develops fulminant hepatitis associated with severe jaundice at about 4 months of age (1). The LEC rats have a gene homologous to the human Wilson's disease gene, ATP7B (2). A defect of the final product (Cu-binding P-type ATPase) of the gene, ATP7b (3), results in abnormal Cu metabolism which is characterized by hepatic Cu accumulation (4, 5). Since a copper-deficient diet prevents occurrence of hepatitis in LEC rats (6), it is thought that accumulation of copper leads to the development of hepatic injury responsible for intracellular copper delivery (2, 7).

It has been reported that iron also accumulates in the liver of LEC rats (8). Furthermore, hepatic iron deprivation prevents spontaneous fulminant hepatitis and liver cancer in LEC rats (9). It is well known that iron can efficiently produce reactive oxygen species (ROS) as well as copper (10, 11). ROS induce several types of DNA damage, such as base alteration and DNA strand breaks (10, 12, 13). It has been reported that the amounts of 8-hydroxydeoxyguanosine (oh^8dG) in DNA, a marker of ROS-derived DNA damage, increase in the liver and kidney of LEC rats at 15 weeks of age, compared with those at 5 and 10 weeks of age, and are 1.8-fold higher than those of control rats (14). However, it remains unknown whether other types of DNA damage are induced in the liver of LEC rats and which accumulation of copper or iron is mainly responsible for induction of DNA damage in the liver cells of LEC rats.

Single-cell gel electrophoresis analysis (comet assay) is a sensitive method for measuring DNA strand breaks (15). As few as one break per 10^{10} Da of DNA can be detected by comet assay (16). Furthermore, the clear advantage of comet assay over other techniques that measure DNA damage is its ability to measure heterogeneity within complex populations (17).

In the present study, we measured DNA strand breaks by comet assay in the liver cells of LEC rats that had been fed standard and low-copper food, and found that hepatic accumulation of copper induced DNA strand breaks in the liver cells, but accumulation of iron did not.

MATERIALS AND METHODS

Rats. Inbred strains of LEC/Hkm (LEC) and WKAH/Hkm (WKAH) rats were cared for according to the principles in the "Guide

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for the Care and Use of Laboratory Animals" prepared by Rakuno Gakuen University. WKAH rats were used as a control in the present study. All rats were maintained under conditions described previously (18). Sixteen males each of LEC and WKAH rats weaned at 4 weeks after birth were fed standard MF-Food (Oriental Yeast Co. Ltd., Tokyo, Japan) containing 7 mg Cu/kg or low-copper food (Clea Japan Inc., Tokyo, Japan) containing 0.1 mg Cu/kg and allowed to drink water *ad libitum*.

Isolation of single cell preparations from the rat liver. Single cells from the rat liver were prepared from 4- to 15-week-old rats as described by Sasaki *et al.* (19). Briefly, small pieces of freshly resected rat liver were homogenized for one stroke in 10 volumes of ice-cold homogenizing buffer (75 mM NaCl, 24 mM EDTA, pH 7.0) with a Potter homogenizer at 700 rpm and centrifuged at 1000*g* for 5 min. The pellets were washed twice with ice-cold homogenizing buffer as described above and resuspended in a small volume of ice-cold homogenizing buffer.

Alkaline single-cell gel electrophoresis assay (comet assay). The comet assay was performed basically according to the method of Singh *et al.* (15) under alkaline conditions with slight modification. Briefly, the isolated liver cells were embedded in 1% low melting-point agarose (Life Technologies, Co. Ltd., Tokyo, Japan) and deposited on top of a 1% agarose base layer (Nakarai Techs Co. Ltd., Osaka, Japan) on the precoating a fully frosted slides (Matsunami Glass Indust. Ltd., Tokyo, Japan). After solidification of the agarose containing approximately 10^4 of the cells, 1% agarose was deposited on the second layer. After solidification of the top-layer agarose, the slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 1% Na-sarcosinate, 10% dimethyl sulfoxide and 1% Triton X-100, pH 10.0) for 1 h at 4°C in a dark room. After lysis, slides were incubated in an electrophoretic buffer (0.3 M NaOH, 1 mM EDTA) for 30 min. Electrophoresis was carried out at 25 V and approximately 400 mA for 25 min at room temperature. The slides were neutralized in 0.4 M Tris–HCl solution (pH 7.5) for 20 min, stained with propidium iodide, and then photographed under a fluorescent microscope (Olympus Co., Japan). Image analyzer software (Rio Grand Software) was used to quantify the different parameters of the images. Generally, 150 images were analyzed per slide. Migration length of nuclei and total length inclusive of nucleus and tail were determined, and then tail length was determined for each cell. Proportion of the cells without tail was normalized in such a way that percentage of the WKAH rat cells without tail at 4 weeks of age was 100.

Measurement of copper and iron contents in the liver. Parts (ca. 0.4 g) of the liver of WKAH and LEC rats were digested with 10 ml of conc. nitric acid and 1 ml of conc. perchloric acid at 80°C until tissues were completely dissolved and then at 120°C for 2 h. The digests were diluted with 0.1 M HNO_3, and the concentrations of copper and iron in the dilutions were determined using a flame type of atomic absorption spectrophotometer (Variant Spectro AA-880).

RESULTS

We examined copper and iron contents in the livers of male LEC and WKAH rats that had been fed standard MF food (Fig. 1). No significant changes were observed in copper contents in the livers of WKAH rats from 4 to 15 weeks of age. Copper contents in the liver of LEC rats were approximately 30-fold higher than those of WKAH rats at 4 weeks of age. Copper accumulated in the liver of LEC rats in an age-dependent manner. No significant differences were observed between copper contents in male and female rats at each week of age from 4 to 15 weeks (data not shown). These

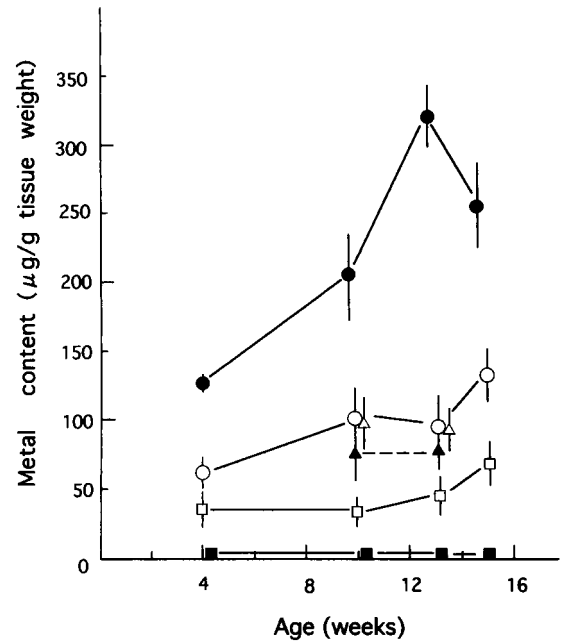

FIG. 1. Contents of copper and iron in the livers of LEC and WKAH rats. Livers were obtained from male LEC rats that had been fed standard MF food (○, ●) or low-copper food (△, ▲) and from male WKAH rats (■, □) from 4 to 15 weeks of age. The copper (●, ■, ▲) and iron contents (○, □, △) (μg/g wet weight of the liver) were determined by atomic absorption spectrophotometry. Points represent the average from four separate experiments. Error bars represent the standard deviation (SD) of the mean values. SD were within symbols at some points.

results were in good agreement with results reported by Lee *et al.* (4). Iron contents in the liver of male LEC rats were significantly higher than those in WKAH rats at each week of age from 4 to 15 weeks, and increased in an age-dependent manner (Fig. 1). When LEC rats were fed low-copper food from 4 weeks of age, copper contents in the liver of LEC rats did not increase at 10 and 13 weeks of age, compared with those at 4 weeks of age, and were significantly lower than those of LEC rats fed standard MF food. In contrast, there was no significant difference in the hepatic iron contents between LEC rats that had been fed standard MF food and low-copper food at 10 and 13 weeks of age. No significant difference was also found in the hepatic iron contents between female LEC rats fed standard MF food and low-copper food at 10 and 13 weeks of age, although the iron contents in the liver of female rats was approximately 2-fold higher than those of male rats older than 10 weeks of age (data not shown).

In comet assay, undamaged DNA remains within the core and broken DNA migrates from the core toward the anode, forming a tail of a comet. In the case of male WKAH rats, no significant differences were found in the proportions of cells without tail among 4-, 10-, 13-, and 15-week-old rat cells (Fig. 2). In contrast, the proportions of male LEC rat liver cells without tail de-

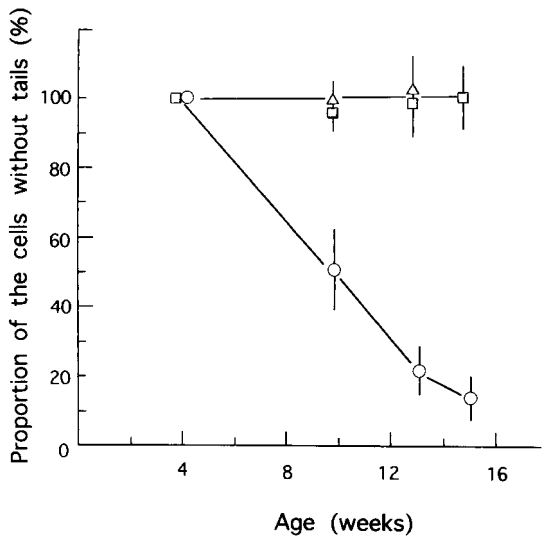

FIG. 2. Proportions of rat liver cells without tail in comet images. Liver cells were prepared from male LEC rats that had been fed standard MF food (○) or low-copper food (△) and from male WKAH rats (□). Proportion of the cells without tail was normalized in such a way that percentage of the WKAH rat cells without tail at 4 weeks of age was 100. Error bars represent SD of the mean values (n = 3–4).

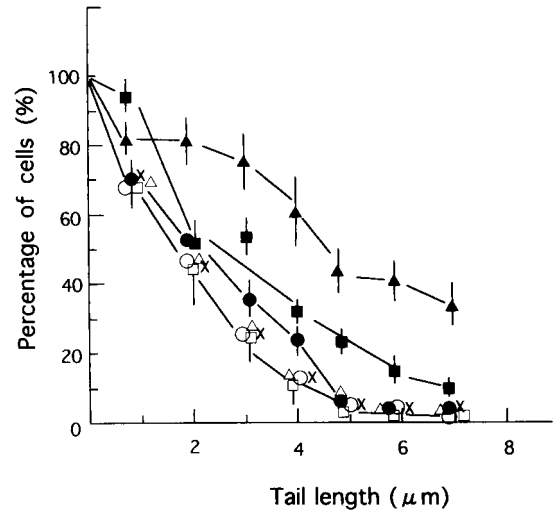

FIG. 3. Distribution of tail lengths of rat liver cells in comet images. Liver cells were prepared from 4-week-old (●), 10-week-old (■), and 13-week-old (▲) male LEC rats fed standard MF food; from 10-week-old (△) and 13-week-old (×) male LEC rats fed low-copper food; and from 4-week-old (○) and 15-week-old (□) male WKAH rats. The proportions of cells with a longer tail than the length indicated in the abscissa to total cell population were plotted as the ordinate. Error bars represent SD of the mean values (n = 3–4). SD were within symbols at some points.

creased in an age-dependent manner from 4 to 15 weeks of age, when LEC rats were fed standard MF food. The proportions of the cells without tail in the liver of LEC rats that had been fed low-copper food at 10 and 13 weeks of age did not decrease, compared with those in the liver of 4-week-old LEC rats and were the same as those of WKAH rats (Fig. 2).

Since it is thought that the cells with longer tails in the comet image contain more breaks in DNA, we measured the tail lengths of each liver cell, and the proportions of rat cells with tail lengths longer than 1 to 7 μm in the total cell population were shown in Fig. 3. In the case of male WKAH rats, there was no difference between the distributions of tail lengths of 4-week-old and 15-week-old rat cells. No significant differences were found in the average tail lengths of 4-, 10-, 13-, and 15-week-old WKAH rat cells (Fig. 4). At 4 weeks of age, no significant differences were observed in the distributions of tail lengths between the liver cells from male LEC and WKAH rats (Fig. 3). When LEC rats were fed standard MF food, the proportions of LEC rat cells with long tails were higher than those of WKAH rat cells from 10 to 15 weeks of age (Fig. 3 and data not shown). The average tail lengths of LEC rat liver cells increased in an age-dependent manner from 4 to 15 weeks of age and significantly longer than those of WKAH rat cells at each week of age (Fig. 4).

The distribution of tail lengths and the average tail lengths of the liver cells from LEC rats that had been fed low-copper food at 10 and 13 weeks of age were the same as those of LEC rats at 4 weeks of age and those of WKAH rats (Figs. 3 and 4). The average tail lengths of liver cells from LEC rats fed low-copper food at 10 and 13 weeks of age were significantly smaller than those of liver cells from LEC rats fed standard MF food. No significant differences were found in the proportion of cells without tail, the distributions of tail lengths,

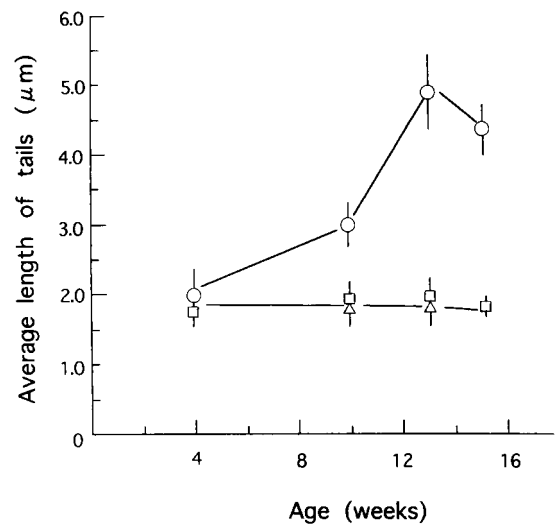

FIG. 4. Average tail lengths of rat liver cells in comet images. Liver cells were prepared from male LEC rats that had been fed MF food (○) or low-copper food (△) and from male WKAH rats (□). The average tail lengths of rat cells in comet images were shown. Error bars represent SD of the mean values (n = 3–4). SD were within symbols at some points.

and the average tail lengths between male and female rats cells at each week of age (data not shown). Accurate analysis of the distribution of tail lengths of liver cells in LEC rats older than 16 weeks was difficult, because LEC rats developed necrotizing hepatic injury (data not shown).

DISCUSSION

A variety of modified comet assays using several parameters have been developed to evaluate the extent of DNA damage (20). In the present study, we used the alkaline comet assay, and the extent of DNA damage was evaluated by the proportion of cells without tail, the distribution of tail lengths and the average tail lengths of comet images. The alkaline version of the comet assay primarily detects single-strand breaks (SSBs) of DNA (26). When LEC rats were fed standard food, the proportions of rat liver cells without tail decreased in an age-dependent manner from 4 to 15 weeks of age. Furthermore, the average tail lengths of LEC rat cells were longer than those of WKAH rats older than 10 weeks of age and increased with age. These results showed that SSBs of DNA were produced in the substantial population of LEC rat liver cells at 10 weeks of age and induced in an age-dependent manner from 10 to 15 weeks of age.

Copper and iron contents in the LEC rat livers were significantly higher than those in the WKAH rat livers, and accumulated in an age-dependent manner in the LEC rat liver. Although copper contents in the livers of LEC rats fed low-copper food were significantly lower than those of LEC rats fed standard food, hepatic iron contents of LEC rats fed low-copper food were almost the same as those of LEC rats fed standard food. The proportions of cells without tail and the average tail lengths in the liver cells from LEC rats fed low-copper food were the same as those of LEC rats at 4 weeks of age and WKAH rats. The results showed that low-copper food prevented induction of SSBs of DNA. Therefore, the present results suggest that the induction of DNA strand breaks in the liver cells of LEC rats is mainly due to accumulation of copper, but not accumulation of iron. Although the hepatic iron contents in female LEC rats were approximately 2-fold higher than those in male rats, no significant differences was observed in comet images between the liver cells of male and female rats (data not shown). The results supported the idea that accumulation of iron was not directly associated with induction of DNA damage in the liver cells of LEC rats. Furthermore, our preliminary results showed that low-iron food did not significantly affect the comet images of LEC rat liver cells at 10 weeks of age, compared with those of LEC rats fed standard food (data not shown). Since it is well known that iron can efficiently produce ROS as well as copper (10, 11), and that ROS induce several types of DNA damage (10, 12, 13), a reason why hepatic accumulation of iron does not produce SSBs of DNA in LEC rat liver cells in the present study remains unclear now.

Although it is well known that SSBs of DNA are rapidly repaired, SSBs are converted into double-strand breaks (DSBs) of DNA when a multiple of SSBs are induced in DNA. DSBs of DNA are thought to be directly associated with cell death unless repaired (21). We have shown that the repair process of DSBs in LEC rat cells is slower than that of WKAH rat cells (22–24). Therefore, DNA strand breaks induced by hepatic copper accumulation may not be repaired efficiently in LEC rat cells. The unrepaired DNA strand breaks may cause extensive cell death which leads to severe hepatic injury, although the present results cannot rule out the possibility that other types of cellular damage induced by copper accumulation, such as lipid peroxidation (25–27), are responsible for development of hepatitis.

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