No Significant Differences Were Observed in the Amounts of DNA Strand Breaks Produced by Copper between Male and Female Liver Cells of Long-Evans Cinnamon (LEC) Strain Rats

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ABSTRACT. The amounts of DNA single strand breaks that are oxidative damage produced by copper were examined by comet assay in the liver cells of an inbred strain of Long-Evans Cinnamon (LEC) rats that spontaneously develops fulminant hepatitis. At 4 weeks of age, copper contents in the liver of LEC rats were approximately 30-fold higher than those of WKAH rats that are control rats used in the present study. Copper accumulated in the liver of LEC rats in an age-dependent manner and no significant differences were observed between copper contents in the livers of males and females at each week of age from 4 to 15 weeks. No significant amounts of DNA strand breaks were found in the liver cells of both male and female WKAH rats from 4 to 15 weeks of age. DNA strand breaks 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. The amounts of DNA strand breaks produced by copper accumulation in the liver cells of female LEC rats are not more abundant than those in the cells of male rats, although it has been reported that hepatitis in female rats is more serious than that in male rats.

KEY WORDS: comet assay, copper, DNA strand break, LEC rat, liver.

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 [23]. A gene responsible for fulminating hepatitis in the LEC rat is \( \text{ATP7b} \), which is homologous to the gene responsible for the human Wilson’s disease, \( \text{ATP7B} \) [16, 20, 21, 29]. A defect of the final product (Cu-binding P-type ATPase) of the gene, \( \text{ATP7b} \) [29], results in abnormal Cu metabolism which is characterized by hepatic Cu accumulation [14, 15]. Since a copper-deficient diet prevents occurrence of hepatitis in LEC rats [27], it is thought that accumulation of copper leads to the development of hepatic injury responsible for intracellular copper delivery [1, 16].

It is well known that copper can efficiently produce reactive oxygen species and that reactive oxygen species induce several types of DNA damage, such as base alteration and DNA strand breaks [13, 28, 30]. It has been reported that the amounts of 8-hydroxydeoxyguanosine in DNA, a marker of reactive oxygen-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 [31]. It has been reported that iron also accumulates in the liver of LEC rats [12]. Although it is known that iron also can efficiently produce reactive oxygen species and induces DNA damage [13, 26], recently we have shown that the copper accumulation in the liver of LEC rats induces DNA strand breaks, but accumulation of iron does not [9].

Clinico-pathological analysis showed that there are sex differences in the characteristics of hepatitis of LEC rats [10]. For instance, the onset of jaundice is a few weeks earlier in females than in males, and 60–70% of the males survive fulminant hepatitis, while only 20% of the females do. It has been reported that hepatic copper concentration in female LEC rats is not higher significantly than those in male LEC rats [17]. However, it remains unclear whether initial yields of oxidative DNA damage induced by copper accumulation in female LEC rat liver cells is different from those in male LEC rat liver cells, and whether sex differences in the characteristics of hepatitis of LEC rats is associated with sex differences in the initial yields of oxidative DNA damage produced by copper accumulation.

In the present study, we measured DNA strand breaks by comet assay in the liver cells of male and female LEC rats, and found that no significant sex differences were observed in the initial yields of oxidative damage of DNA in the liver cells.

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 for the Care and Use of Laboratory Animals’ prepared by Rakuno Gakuen University. All rats were maintained under conditions described previously [4]. WKAH rats were used as a control in the present study. Sixteen males and 16 females each of LEC and WKAH rats...
weaned at 4 weeks after birth were fed standard MF-Food (Oriental Yeast Co., Ltd., Tokyo, Japan) and allowed to drink water ad libitum.

Isolation of single cell preparations from the rat liver:
Single cells from the rat liver for comet assay were prepared from 4- to 15-week-old rats as described by Sasaki et al. [24]. 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 1,000 × 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. [25] under alkaline conditions with slight modifications. 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 (Nacarai Tesque 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% TritonX-100, pH 10.0) for 1 hr 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, 100 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 (c.a. 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 hr. The digestes were diluted with 0.1 M HNO₃, and the concentrations of copper and iron in the dilutions were determined using a flame type of atomic absorption spectrophotometer ( Variant Spectro AA-880).

Statistical analysis: All data are expressed as means ± standard error.

RESULTS

When copper contents in the livers of LEC and WKAH rats were examined, no significant changes were observed in copper contents in the livers of WKAH rats from 4 to 15 weeks of age (3.73–4.92 µg Cu/g wet weight), and no significant differences were found in copper contents between male and female WKAH rats at each week of age (Fig. 1). Copper contents in the liver of LEC rats were approximately 30-fold higher (127.1 ± 8.3 and 131.6 ± 10.9 µg Cu/g wet weight for males and females, respectively) than those of WKAH rats at 4 weeks of age. Copper accumulated in the liver of LEC rats in an age-dependent manner (Fig. 1). No significant differences were observed in copper contents between male and female LEC rats at each week of age from 4 to 15 weeks.

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. No significant differences were found in the proportions of cells without tail among 4-, 10-, 13- and 15-week-old rat liver cells in both male and female WKAH rats (Fig. 2). The proportions of LEC rat liver cells without tail decreased in an age-dependent manner from 4 to 15 weeks of age. No significant differences were observed

![Fig. 1. Contents of Cu in the livers of LEC and WKAH rats. Livers were obtained from female ( ) and male ( ) LEC rats, and from female ( ) and male ( ) WKAH rats from 4 to 15 weeks of age. The copper 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 of the mean values. Standard deviations were within symbols at some points.](image-url)
DNA STRAND BREAKS IN LIVER OF LEC RATS BY CU

Since it is thought that the cells with longer tails in the comet image contain more breaks in DNA, we measured the average tail lengths of comet image of the liver cells. The average tail lengths of 4-week-old male and female WKAH rat cells were 1.72 ± 0.19 and 1.93 ± 0.22 µm, respectively. No significant differences were found in the average tail lengths of 4-, 10-, 13- and 15-week-old male and female WKAH rat cells (Fig. 3). At 4 weeks of age, the average tail lengths of comet image of LEC rat cells were 2.00 ± 0.19 and 2.29 ± 0.30 µm for males and females, respectively and slightly longer than those of WKAH rat cells. The average tail lengths of LEC rat liver cells increased in an age-dependent manner from 4 to 15 weeks of age, and were significantly longer than those of WKAH rat liver cells at each week of age, except those of female LEC rat cells at 10 weeks of age (Fig. 3). The average tail lengths of female rat liver cells in comet images were almost the same as those of male LEC rat cells at each week of age from 4 to 15 weeks except at 10 weeks of age.

DISCUSSION

Single-cell gel electrophoresis analysis (comet assay) is a sensitive method for measuring DNA strand breaks [25]. As few as one break per 10^10 Da of DNA can be detected [2]. Furthermore, the clear advantage of comet assay over other techniques that measure DNA strand breaks is its ability to measure heterogeneity within complex populations [20]. A variety of modified comet assays using several parameters have been developed to evaluate the extent of DNA strand breaks [19]. The alkaline version of comet assay primarily detects single-strand breaks (SSBs) of DNA [19]. In the present study, we used the alkaline comet assay, and the extent of SSBs of DNA was evaluated by the proportions of the cells without tail and the average tail lengths of comet images. The proportions of LEC 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 for males and 13 weeks of age for females, 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. The reason why the average tail lengths of male LEC rat cells were significantly longer than those of female LEC rat cells at 10 weeks of age remains unclear.

Copper contents in the LEC rat livers were significantly higher than those in the WKAH rat livers, and copper accumulated in an age-dependent manner in the LEC rat liver. The previous report showed that accumulation of copper induces SSBs of DNA in the liver cells of LEC rats [9]. Although copper contents in the livers of 4-week-old LEC rats were approximately 30-fold higher than those of WKAH rats, the proportions of the cells without tail and the average tail lengths in comet images of the liver cells from LEC rats at 4 weeks of age were not significantly different from those of WKAH rats. These results suggest that there may be a threshold for induction of DNA strand breaks at copper contents more than 130 µg/g wet weight of the liver.
It has been reported that hepatitis in LEC rats is closely associated with copper toxicity [3, 14, 17] and that a copper-deficient diet prevents occurrence of hepatitis in LEC rats [27] and induction of DNA strand breaks in the LEC rat liver cells [9]. Sex differences have been reported in occurrence of fulminant hepatitis in LEC rats [10]. Generally speaking, hepatitis in female rats is more serious than that in male rats. However, in the present study no sex differences were observed in the hepatic copper contents and the amounts of DNA strand breaks in the LEC rat liver cells. These results suggest that the amounts of DNA strand breaks produced by copper is not be directly responsible for the sex difference in the characteristics of fulminant hepatitis in LEC rats. Oxidative DNA damage produced by copper may initiate the process of hepatitis, but biological factors other than the oxidative damage may play an important role in the sex difference in occurrence of hepatitis.

Since it has been reported that sex hormones do not influence the occurrence of fulminant hepatitis but do influence mortality due to hepatitis [11], sex hormones may play an important role in the sex difference of late phase of hepatitis.

Other characteristics of LEC rats are a high incidence of spontaneous liver cancer in long-surviving individuals [32] and an increased sensitivity to a variety of DNA damaging agents in vivo and in vitro [4–6, 18]. Furthermore, LEC rat cells show abnormalities of transient cell cycle arrests from the G1 to S phase and at the G2/M phase after treatment of the cells with DNA-damaging agents [7, 8]. Many studies suggest that abnormalities of cell-cycle checkpoint can contribute to the process of neoplastic transformation of the cells. Therefore, DNA damage induced by copper and the abnormal cell-cycle check point may be associated with hepatocarcinogenesis in LEC rats. Thus, LEC rats could provide a useful animal model to assist in understanding the mechanism of human Wilson’s disease, genotoxicity of copper and hepatocarcinogenesis.

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REFERENCES


