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Elevated Level of 8-Hydroxydeoxyguanosine in DNA of Liver, Kidneys, and Brain of Long-Evans Cinnamon Rats

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Long-Evans Cinnamon (LEC) rats, a mutant strain originating from Long-Evans rats, spontaneously develop hereditary hepatitis followed by hepatocellular carcinoma. The hepatic disorder in LEC rats is associated with their abnormal copper metabolism; metal-catalyzed reactions often give rise to oxygen radicals, which may be related to the carcinogenesis. By means of high-pressure liquid chromatography with electrochemical detection, cellular DNA damage caused by oxygen radicals can be assessed in terms of the amount of 8-hydroxydeoxyguanosine (oh^{8}dG). We assayed the amount of oh^{8}dG in DNA of liver, kidneys, and brain of LEC and Long-Evans Agouti (LEA) control rats in seven groups (n=3 to 6) aged from 5 weeks to 24 months. Control rats, a healthy sibling line, were age-matched. The amount of oh^{8}dG was correlated with the severity of the age-related clinical symptoms in LEC rats. The amount was higher in LEC rats than in the controls, especially in the liver at the acute stage of hepatitis. These findings suggest that oxygen radicals may be important in the carcinogenesis that occurs in LEC rats.

Key words: Hepatocellular carcinoma — 8-Hydroxydeoxyguanosine — Long-Evans Cinnamon rat — Oxygen radical — Carcinogenesis

Long-Evans Cinnamon (LEC[7]) rats are an inbred strain established from Long-Evans rats at the Center for Experimental Plants and Animals, Hokkaido University.[1)] About 4 months after birth, there is a sudden onset of acute hepatitis in these rats; the disease resembles human fulminant hepatitis clinically and histopathologically.[2)] About half of the rats die within 2 weeks after the onset of hepatitis, followed by renal failure. The remaining rats survive with chronic hepatitis for longer than 1 year and develop hepatocellular carcinoma.[3)] LEC rats accumulate copper in the liver, and their serum copper and ceruloplasmin concentrations are low, as in human Wilson's disease.[4)] The similarity suggests a close relationship of the hepatitis to copper toxicity. It is likely that in LEC rats, the abnormal copper metabolism has some as-yet undefined relationship to carcinogenesis.[5)]

8-Hydroxydeoxyguanosine[8] (oh^{8}dG) is one of the main DNA modifications produced by active oxygen species, which are generated by both environmental carcinogens and normal aerobic metabolism.[6)] By measuring oh^{8}dG in cellular DNA *in vivo*, we can estimate the extent of DNA damage caused by oxygen radicals. Oxygen radicals can be generated in the presence of metal ions.[7–9)] In this study, we assayed the concentration of oh^{8}dG in DNA of liver, kidneys, and brain of LEC rats of various ages, using a sensitive electrochemical detector (ECD) coupled with high-pressure liquid chromatography (HPLC).[10)] Formation of oh^{8}dG in cellular DNA *in vivo* was easily analyzed by this method.

MATERIALS AND METHODS

Animals LEC rats and Long-Evans Agouti (LEA) rats were provided by the Center for Experimental Plants and Animals, Hokkaido University. LEA rats are a healthy sibling line of LEC rats, and oh^{8}dG concentrations in tissues of LEA rats were used as controls. LEC rats were examined when they were 5, 10, 15, 26, or 40 weeks, or 12 or 24 months of age, but 26-week-old and 24-month-old control rats were not examined.

Tissue homogenates The animals were killed under ether anesthesia. The liver, kidneys, and brain were quickly

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[7] The abbreviations used are: LEC, Long-Evans Cinnamon; dG, deoxyguanosine; ECD, electrochemical detector; HPLC, high-pressure liquid chromatography; LEA, Long-Evans Agouti; oh^{8}dG, 8-hydroxydeoxyguanosine.

[8] The favored tautomeric structure of 8-hydroxydeoxyguanosine is the 8-keto form, 7,8-dihydro-8-oxodeoxyguanosine.

removed and stored at $-70°C$ until use. A portion of each sample (200–400 mg) was homogenized in a Potter-Elvehjem homogenizer with 1 ml of buffer that contained 0.1 *M* EDTA (pH 8.0) and 0.15 *M* NaCl.

DNA extraction and enzymatic digestion DNA was extracted from the homogenates with a nucleic acid extractor (model 340; Applied Biosystems, Foster City, CA). The extracted DNA (about 100 μg) was dissolved in 100 μl of water and denatured by being heated at 100°C for 3 min. To the solution of denatured DNA, 2 μl of 1 *M* sodium acetate (pH 4.8) and 4 μg of nuclease P1 (5 mg/ml, Seikagaku Kogyo, Tokyo) were added, and then the mixture was digested into nucleotides at 37°C for 1 h. To each sample, 16 μl of 1 *M* Tris-HCl (pH 7.2) was added and the mixture was treated with 1.3 units of alkaline phosphatase (type III from *Escherichia coli*; Sigma Chemical Co., St. Louis, MO) at 37°C for 1 h.

Analysis of oh^{8}dG The oh^{8}dG in the digested DNA was assayed by HPLC with ECD. A column (Ultrasphere ODS column; 5 μm, 4.6 mm$\times$25 cm, Beckman Instrument, Fullerton, CA) was connected to an HPLC apparatus (CCPM; Tosoh Co., Tokyo) coupled with an ECD (model 5100A; ESA, Inc., Bedford, MA) equipped with an analytical cell (model 5010; detector 1: 0.15 V, detector 2: 0.30 V) and a guard cell (model 5020; 0.35 V). The column was eluted with 10 m*M* NaH_2PO_4 containing 8% methanol at the flow rate of 1.0 ml/min. The amount of deoxyguanosine (dG) was calculated from the absorbance at 290 nm measured with a UV monitor (model UV-8000; Tosoh Co.), while the amount of oh^{8}dG was simultaneously assayed by the ECD. The amount of oh^{8}dG was expressed as the ratio of the peak height of oh^{8}dG to the peak height$\times 10^5$ of dG.

Statistical analysis All results are expressed as means$\pm$SD for three to six rats. The differences were analyzed for statistical significance by using Student's *t* test.

RESULTS

The overall mean liver oh^{8}dG content of all age groups was higher in LEC rats ($P<0.05$) than in the controls, and significance was especially high at 15 weeks ($P<0.005$) and 40 weeks ($P<0.01$) compared with age-matched controls (Table I). The mean kidney oh^{8}dG content was higher in 15-week-old and 12-month-old LEC rats (both $P<0.05$) than in age-matched controls. The mean brain oh^{8}dG content tended to be higher in 40-week-old and 12-month-old LEC rats than in age-matched controls, but without significant differences.

Among the animals of different ages, the 15-week-old LEC rats had the highest oh^{8}dG level in the liver and kidneys. The liver and kidney oh^{8}dG levels were higher at this age than in older groups (both $P<0.01$). Brain oh^{8}dG reached a peak at about 40 weeks of age; the amount at that age was significantly higher than in the other age groups (all $P<0.01$).

DISCUSSION

oh^{8}dG is an important oxidative product of cellular DNA, and its assay provides information about oxidative stress associated with aging, carcinogenesis, and mutagenesis.[11-16] *E. coli* has a repair mechanism for oh^{8}dG in cellular DNA.[17, 18] Findings about the mutM, mutT, and mutY mutants of *E. coli* indicate that oh^{8}dG in DNA and oh^{8}dG triphosphate in the nucleotide pool are directly involved in spontaneous mutation in this bacterium.[19-21] Mammals also have repair enzymes for oh^{8}dG in cellular DNA.[22, 23] The finding that copper chelating agents prevent the alteration of dG to oh^{8}dG suggests formation of oh^{8}dG in a copper-catalyzed reaction.[24]

The changes in the oh^{8}dG content we observed corresponded with the clinicopathological stage of disease in

Table I. Amount of oh^{8}dG in DNA of Liver, Kidneys, and Brain of LEC and LEA Rats at Different Ages

Age	oh^{8}dG/10^{5}dG[a]					
	Liver		Kidney		Brain	
	LEC	LEA	LEC	LEA	LEC	LEA
5 wk	0.99±0.11	1.37±0.33	1.25±0.65	0.64±0.10	1.09±0.30	1.19±0.47
10 wk	1.31±0.30	1.28±0.51	1.33±1.00	1.13±0.67	2.04±0.57	2.22±0.51
15 wk	5.56[b]±0.66	3.06±1.33	4.97[d]±0.15	2.55±1.13	1.74±1.34	1.49±0.69
26 wk	2.17±1.11		2.07±0.15		1.50±0.28	
40 wk	2.34[c]±0.70	1.32±0.18	1.78±0.39	2.12±1.11	3.79±0.85	1.86±0.67
12 mo	1.99±0.84	1.03±0.03	1.78[d]±0.47	1.04±0.05	1.83±0.83	1.09±0.24
24 mo	1.69±0.27		2.15±0.88		1.25±0.11	

a) Amount of oh^{8}dG is expressed as the ratio to 10^5 dG (mean±SD, n=3 to 6).
b), *c*), *d*) Significantly different from the age-matched LEA rats at $P<0.005$, $P<0.01$, and $P<0.05$, respectively.
wk, weeks; mo, months.

LEC rats. That is, liver and kidney oh^8dG peaked at about 15 weeks, when acute hepatitis begins, and decreased later, when symptoms of the surviving rats stabilize. At about 40 weeks of age, some LEC rats develop neurological symptoms such as convulsions. We observed the highest level of brain oh^8dG at 40 weeks.

The copper concentration may be associated with the oh^8dG concentration, so whether there is a correlation between these concentrations is of interest. In the liver of LEC rats, the oh^8dG levels we found seemed to correspond with the copper concentration. LEC rats have more copper in the liver at 3 months of age than at other ages.[5] In the brain of LEC rats, oh^8dG levels also corresponded with the copper concentration. The copper concentration in the brain of 8-month-old LEC rats is higher than in the 3-month-old rats.[4] In the kidney of LEC rats, oh^8dG levels did not correspond with the copper concentration. The copper concentration of 3-month-old LEC rats is lower than that of the 8-month-old rats.[4] It seems that the oh^8dG level in the kidneys of these rats is influenced by the destructive changes involved in renal failure.

There is some question as to why oh^8dG levels decreased to near the normal range after reaching a peak. One possibility is that the copper concentration may change together with the oh^8dG levels in the liver and brain. Still, the lowest copper concentration in the liver of LEC rats is 3 to 60 times that of LEA rats.[5] Other possibilities are that the repair mechanisms for oh^8dG may become more active than before, or that the levels of metal-ion-related proteins such as ceruloplasmin or metallothionein may become higher than before, reducing the number of free copper ions.[25]

It is difficult to relate the high incidence of hepatocellular carcinoma in LEC rats to the high level of oxygen radicals, because the oh^8dG level was higher not only in the liver but also in the kidneys of LEC rats. However, unlike renal cells, hepatocytes can regenerate fully actively. Oxidative DNA damage seems to be involved in carcinogenesis in the liver only. This reasoning and our observation of a peak of liver oh^8dG that coincided with the onset of acute hepatitis suggest that oxygen radicals generated at this time may help to initiate hepatitis and further carcinogenesis. This carcinogenesis in LEC rats might be related to copper toxicosis mediated by oxygen radicals; so far, copper toxicosis has been discussed in association with experimental cirrhosis of the liver in Wistar rats[26] and in the hepatitis that spontaneously occurs in LEC rats.[4,5] The presence of orcein-positive hepatocellular material, which seems to be a morphologic counterpart of copper-protein complexes, in primary liver tumors including hepatocellular carcinomas[27] suggests a mechanism of copper-related carcinogenesis in the liver, as does the high copper concentration in sera of patients with hepatocellular carcinoma compared with patients with cirrhosis.[28]

The activity of the drug-metabolizing enzymes in hepatocytes in LEC rats changes abnormally,[29] and LEC rats are unusually sensitive to several hepatocarcinogens.[30] A number of factors including oxygen radicals may participate in carcinogenesis in LEC rats.

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