

Role of Copper Accumulation in Spontaneous Renal Carcinogenesis in Long-Evans Cinnamon Rats

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Spontaneous renal cell tumors in totals of 223 male and female Long-Evans Cinnamon (LEC) rats of 51–120 weeks old, 157 male F344 rats of 51–120 weeks old, and 14 male Long-Evans Agouti (LEA) rats of 51–70 weeks old were examined histologically. The incidences of renal cell tumors increased with age in male and female LEC rats, but no tumors developed in F344 or LEA rats. Dilated atypical tubules of the kidneys were observed at high incidence in aged LEC rats. Copper staining of LEC rat kidneys showed a positive reaction in proximal tubules of the cortex and the outer stripe of the medulla. The renal copper concentration of LEC rats reached a peak in the period of necrotizing hepatitis with renal tubular necrosis, and was higher than that in F344 rats for up to 106 weeks. In contrast, the renal iron concentration of LEC rats was lower than that in F344 rats except in the period of necrotizing hepatitis. Long-term treatment of LEC rats with D-penicillamine, a copper-chelating agent, inhibited accumulation of copper, but not iron, in the kidneys, and inhibited the development of karyomegaly of proximal tubules and dilated atypical tubules. These results suggest that persistent copper accumulation after toxic necrosis of tubules is the major cause of spontaneous renal carcinogenesis in LEC rats.

Key words: Renal carcinogenesis — Spontaneous — Copper — LEC rat

The LEC rat is an inbred strain showing abnormally high copper accumulation in the liver,¹⁾ and has a deletion in the copper-transporting ATPase gene (*Atp7b*)²⁾ homologous to the human Wilson's disease gene. This mutant rat strain was originally isolated from a closed colony of Long-Evans rats.³⁾ LEC rats develop necrotizing hepatitis with jaundice 4 to 6 months after birth and this hepatitis is inherited in an autosomally recessive manner.⁴⁾ About 20–50% of these rats die of fulminant hepatitis, and hepatocellular carcinomas develop in rats of 12 months old or more that recover from the liver injury.^{4–6)} Both the hepatitis and hepatocellular carcinomas can be prevented by treatment with the copper-chelating agent, D-penicillamine or trientine.^{7,8)}

Patients with Wilson's disease are known to have abnormal renal function and penicillamine therapy improves their renal function,⁹⁾ but we know of no reports of renal neoplasms in cases of Wilson's disease. We have reported that renal cell tumors develop spontaneously in LEC rats.¹⁰⁾ In the present study, we examined preneoplastic and neoplastic lesions of the kidneys in rats of 51–120 weeks old, and the changes in renal copper and iron con-

centrations of LEC and F344 rats of 6–106 weeks old. We also investigated the effect of D-penicillamine on spontaneous renal carcinogenesis.

MATERIALS AND METHODS

Animals LEC/Tj rats and LEA/Tj rats, a sibling line of the LEC rats, were bred in the Institute for Animal Experimentation of the University of Tokushima, in specific pathogen-free conditions. In our laboratory, the mortality rate of LEC rats during the period of jaundice is 12.4% for males ($n=193$) and 42.3% for females ($n=52$). F344/DuCrj rats were obtained from Charles River Japan, Inc., Kanagawa. Animals were housed three to a plastic cage with sterilized woodchips for bedding in an air-conditioned room at $23\pm 2^\circ\text{C}$ and $55\pm 10\%$ humidity with a 12 h light/dark cycle, and given pellet diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*.

Spontaneous renal cell tumors For examining spontaneous renal cell tumors, we used totals of 186 male and 37 female LEC rats of 51–120 weeks old, 157 male F344 rats of 51–120 weeks old, and 14 male LEA rats of 51–70 weeks old. All rats were untreated controls used for carcinogenicity studies or kept for spontaneous tumorigenesis studies in our laboratory over the last 6 years. Therefore, the number of aged LEC rats was limited. Animals were killed by cervical arteriotomy under ether anesthesia and blood was removed by aortotomy. One horizontal slice of

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Abbreviations: LEC, Long-Evans Cinnamon; LEA, Long-Evans Agouti; BrdU, 5-bromo-2'-deoxyuridine; 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

each kidney, and other major organs were fixed in 10% buffered formalin (pH 7.4), embedded in paraffin, sectioned and stained with hematoxylin and eosin for histological examination. In addition, the liver and kidneys of selected animals were stained for copper deposition by a modification of Timm's method.¹¹⁾ Histological types of renal cell tumors were classified as reported.¹²⁾

BrdU labeling For examination of cell proliferating activity in the kidney, six 57-week-old male LEC rats were given an i.p. implantation with an osmotic minipump (Alzet, model 2001; Alza Corp., Palo Alto, CA) of 120 mg/ml BrdU (Sigma Chemical Co., St. Louis, MO). Then 7 days later, they were killed, and their kidneys were fixed in 95% ethanol. Paraffin-embedded sections were treated first with 4 N hydrochloric acid for 20 min, then with boric acid-borax buffer (pH 7.6) for 10 min, and then with 0.05% protease for 1 min. A mouse anti-BrdU monoclonal antibody (Becton Dickinson, Mountain View, CA) was used as the primary antibody. For immunostaining, a DAKO LSAB kit, peroxidase (DAKO Co., Carpinteria, CA) was used as recommended by the manufacturer.

Analyses of copper and iron concentrations Tissues of the kidneys of LEC and F344 rats in weeks 6, 20, 36, 64 and 106 were stored at -80°C until use. They were ashed with nitric acid, and their copper and iron concentrations were measured in an atomic absorption spectrophotometer (AA-782, Nippon Jarrel Ash, Co., Kyoto).

Effect of D-penicillamine on the development of renal lesions Male 6-week-old LEC rats were divided into two groups (n=14). The experimental group was given tap water containing 0.07% D-penicillamine (Tokyo Chemical Industry, Tokyo) 5 days a week for 56 weeks, while the control group was given water only. D-Penicillamine solution was prepared every other day. In week 56, all surviving animals were killed and their livers and kidneys were examined histologically. The metal concentrations of these organs (n=6) were also measured.

Statistical analyses Data on copper and iron concentrations in the kidney were analyzed by using Student's *t* test and the incidences of preneoplastic and neoplastic lesions were analyzed by means of Fisher's exact probability test.

RESULTS

Spontaneous liver tumors The incidences of liver tumors in LEC, F344 and LEA rats are shown in Table I. Hepatocellular tumors (hepatocellular adenomas plus carcinomas) developed in most LEC rats of over 71 weeks. Hepatocellular tumors were very rare in F344 rats until 120 weeks old, and were absent in LEA rats of up to 70 weeks old.

Spontaneous kidney tumors and dilated atypical tubules Renal cell adenomas were microscopic in size. Fig. 1A shows an intracystic renal cell adenoma. Renal cell carcinomas were larger than adenomas and characterized by areas of necrosis, sarcomatous change and invasive growth (Fig. 1B). All gross renal cell carcinomas developed in male LEC rats, and their diameters were 35 mm (63 weeks), 6 mm (65 weeks), 13 mm (74 weeks), 15 mm (79 weeks), 15 mm (82 weeks), 10 mm (99 weeks), 2 and 4 mm (105 weeks), 8 mm (111 weeks), 15 mm (112 weeks), and 30 mm (113 weeks). No mesenchymal or embryonal tumors were observed in this study. The incidence of renal cell tumors (renal cell adenomas plus carcinomas) increased with age in both sexes, those in male and female LEC rats being 8/147 (5%) in weeks 51-70, 5/37 (14%) in weeks 71-90, 4/24 (17%) in weeks 91-110, and 8/15 (53%) in weeks 111-120. No tumors developed in F344 or LEA rats during the observation periods (Table II).

Massive renal tubular necrosis occurred during the period of jaundice (Fig. 1C). Karyomegaly of the proximal tubules was observed in all LEC rats examined (Fig. 2A). Copper staining of the kidneys of LEC rats demonstrated copper deposition in the proximal tubules in the

Table I. Incidences of Spontaneous Hepatocellular Tumors^{a)} in LEC, F344 and LEA Rats

| Strain | Sex | No. of rats | Age (weeks) | | | | | | |
|--------|-----|-------------|--------------------------|---------------------------|---------------------------|-------------------------|----------------------------|----------------------------|------------------------------|
| | | | 51-60 | 61-70 | 71-80 | 81-90 | 91-100 | 101-110 | 111-120 |
| LEC | M | 186 | 7/46 (15%) ^{e)} | 32/71 (45%) ^{d)} | 21/27 (78%) ^{d)} | 6/7 (86%) ^{c)} | 10/10 (100%) ^{c)} | 12/12 (100%) ^{c)} | 13/13 (100%) ^{b,c)} |
| | F | 37 | — | 13/30 (43%) | 2/2 (100%) | 1/1 (100%) | 1/2 (50%) | — | 2/2 (100%) |
| F344 | M | 157 | 0/42 | 0/9 | 0/4 | 1/38 (3%) | 0/24 | 0/4 | 0/36 |
| LEA | M | 14 | 0/11 | 0/3 | — | — | — | — | — |

a) Hepatocellular adenomas plus carcinomas.

b) Two rats with hepatocellular carcinomas had metastases to the lung and adrenal gland, respectively.

c-e) Significantly different from F344 rats by Fisher's exact probability test: c) P<0.001, d) P<0.01, e) P<0.02.

cortex and the outer stripe of the medulla (Fig. 3). Dilated atypical tubules lined by single or multiple layers of acidophilic cells (Fig. 1D, 1E) were found in more than

half the LEC rats of more than 81 weeks old, but only in one F344 rat (Table III). Some of the proliferating lining cells of the dilated atypical tubules stained positively

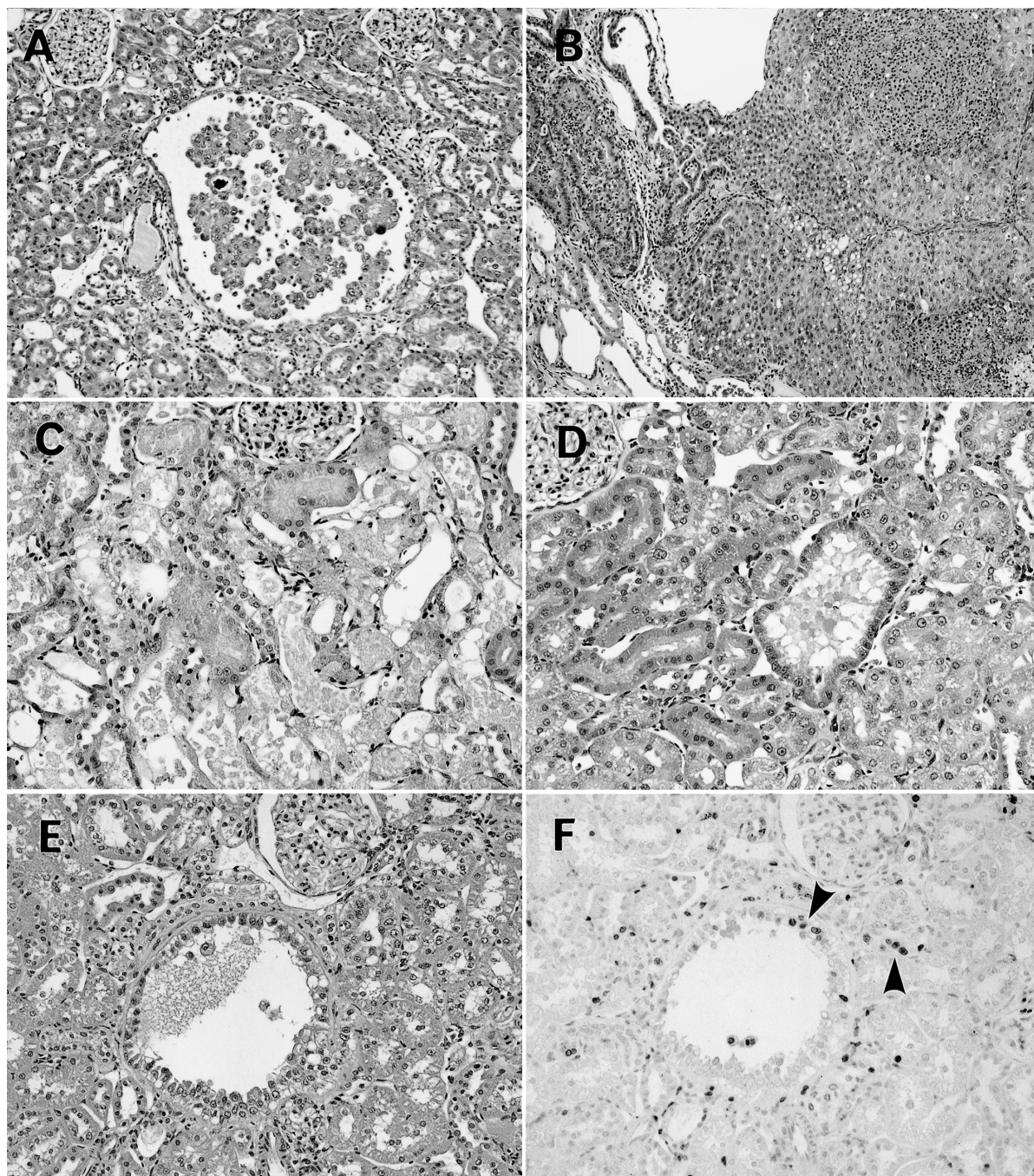


Fig. 1. Histological and immunohistochemical appearances of kidney of LEC rats. (A) Intracystic renal cell adenoma in a 56-week-old male rat (H & E, $\times 99$), (B) renal cell carcinoma in a 112-week-old male rat (H & E, $\times 99$), (C) tubular necrosis in a 17-week-old male rat (H & E, $\times 150$), (D) dilated atypical tubule in a 65-week-old female rat (H & E, $\times 150$), (E) dilated atypical tubule with mild epithelial hyperplasia in a 57-week-old male rat (H & E, $\times 150$), (F) BrdU-positive cells in a serial section (arrowhead, $\times 150$).

Table II. Incidences of Spontaneous Renal Cell Tumors in LEC, F344 and LEA Rats

| Strain | Sex | No. of rats | Histology | Age (weeks) | | | | | | |
|--------|-----|-------------|--------------------------------|-------------|------------|------------|-----------|------------|------------|----------------------------|
| | | | | 51-60 | 61-70 | 71-80 | 81-90 | 91-100 | 101-110 | 111-120 |
| LEC | M | 186 | renal cell tumor ^{a)} | 2/46 (4%) | 3/71 (4%) | 3/27 (11%) | 1/7 (14%) | 2/10 (20%) | 2/12 (17%) | 6/13 (46%) ^{b,c)} |
| | | | adenoma | 2 (4%) | 1 (1%) | 1 (4%) | 0 | 1 (10%) | 1 (8%) | 4 (31%) ^{d)} |
| | | | carcinoma | 0 | 2 (3%) | 2 (7%) | 1 (14%) | 1 (10%) | 1 (8%) | 4 (31%) ^{d)} |
| LEC | F | 37 | renal cell tumor | — | 3/30 (10%) | 1/2 (50%) | 0/1 | 0/2 | — | 2/2 (100%) |
| | | | adenoma | — | 3 (10%) | 0 | 0 | 0 | — | 2 (100%) |
| | | | carcinoma | — | 0 | 1 (50%) | 0 | 0 | — | 0 |
| F344 | M | 157 | renal cell tumor | 0/42 | 0/9 | 0/4 | 0/38 | 0/24 | 0/4 | 0/36 |
| LEA | M | 14 | renal cell tumor | 0/11 | 0/3 | — | — | — | — | — |

a) Renal cell adenoma plus renal cell carcinoma.

b) Two rats had both adenoma and carcinoma.

c, d) Significantly different from F344 rats by Fisher's exact probability test: c) $P < 0.001$, d) $P < 0.01$.

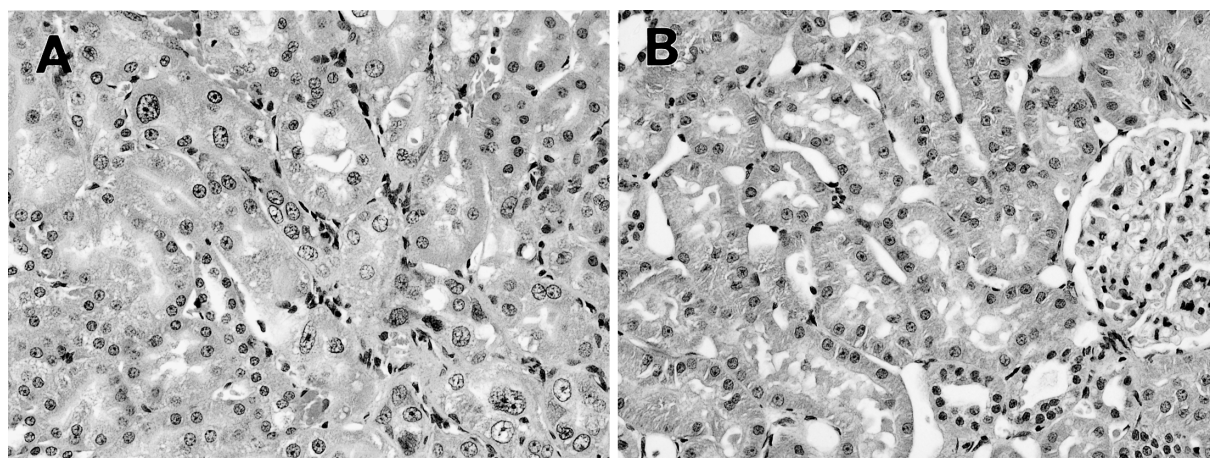


Fig. 2. Proximal tubules of 62-week-old untreated and D-penicillamine-treated LEC rats (H & E, $\times 198$). (A) Karyomegaly in an untreated rat, (B) no apparent karyomegaly in a D-penicillamine-treated rat.

with BrdU (Fig. 1F). So-called nephropathy, which is often observed in aged F344 rats, was absent or mild in LEC rats.

Copper and iron concentrations in the kidneys The copper concentration in the kidneys of LEC rats increased dramatically in week 20 when necrotizing hepatitis occurred to $170 \pm 30 \mu\text{g/g}$ wet weight, which was 24 times that in F344 rats ($7 \pm 1 \mu\text{g/g}$, Fig. 4). Its concentration decreased markedly in week 36 ($26 \pm 3 \mu\text{g/g}$), but was still significantly higher than that in F344 rats in weeks 64 and 106. The iron concentration in the kidneys of LEC rats also reached a peak in week 20 ($140 \pm 41 \mu\text{g/g}$) and then decreased gradually. On the other hand, the iron concentration in F344 rats increased with age. After about 1

year, the iron concentration in F344 rats became higher than that in LEC rats.

Effect of D-penicillamine Two rats which died of unknown causes in week 55 (untreated) and in week 45 (D-penicillamine-treated) were excluded from the effective numbers (Table IV). Karyomegaly of proximal tubules, dilated atypical tubules and renal cell adenoma developed in untreated LEC rats, whereas none of these renal lesions developed in D-penicillamine-treated rats (Fig. 2). The incidence of liver tumors was also decreased in D-penicillamine-treated rats. On D-penicillamine treatment, the copper concentration in the liver was decreased at 56 weeks, but the iron concentration was increased significantly (Fig. 5). Copper concentration in the kidneys was

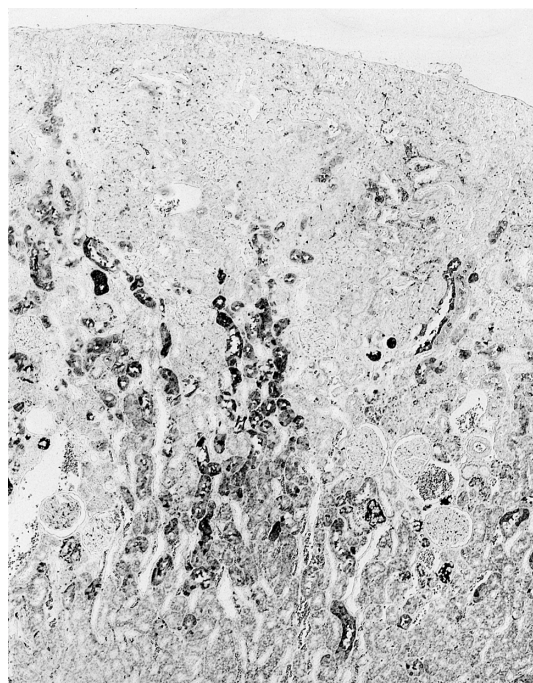


Fig. 3. Copper staining of the kidney of a 62-week-old male LEC rat. Note copper deposits in proximal tubular cells in the cortex and the outer stripe of the medulla (modified Timm's method, $\times 40$).

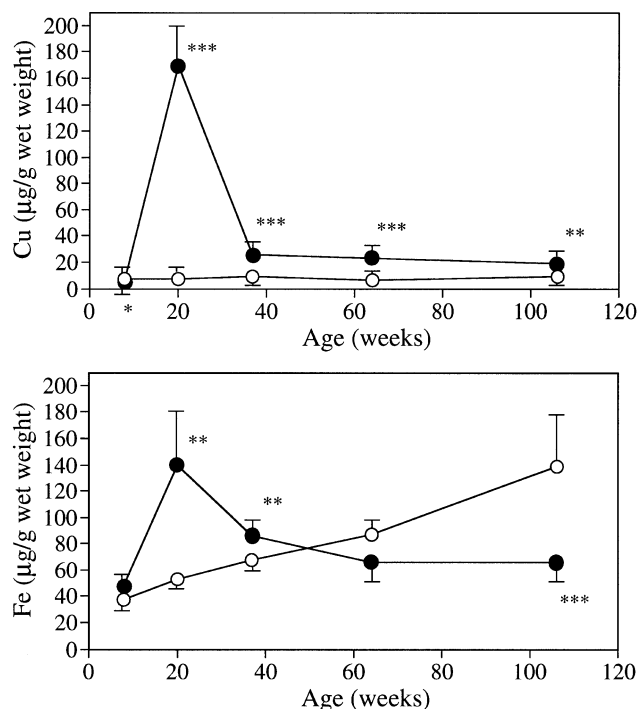


Fig. 4. Copper and iron concentrations in the kidneys of LEC and F344 rats ($n=5$ to 6). Differences are significant at * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ by Student's t test. ● LEC, ○ F344.

Table III. Incidences of Dilated Atypical Tubules of the Kidneys in LEC, F344 and LEA Rats

| Strain | Sex | No. of rats | Age (weeks) | | | | | | |
|--------|-----|-------------|-------------|-------------|-------------|-------------------------|--------------------------|---------------------------|----------------------------|
| | | | 51-60 | 61-70 | 71-80 | 81-90 | 91-100 | 101-110 | 111-120 |
| LEC | M | 186 | 5/46 (11%) | 19/71 (27%) | 12/27 (44%) | 4/7 (57%) ^{a)} | 6/10 (60%) ^{a)} | 11/12 (92%) ^{a)} | 13/13 (100%) ^{a)} |
| | F | 37 | — | 10/30 (33%) | 2/2 (100%) | 1/1 (100%) | 1/2 (50%) | — | 2/2 (100%) |
| F344 | M | 157 | 0/42 | 0/9 | 0/4 | 0/38 | 0/24 | 0/4 | 1/36 (3%) |
| LEA | M | 14 | 0/11 | 0/3 | — | — | — | — | — |

a) Significantly different from F344 rats at $P<0.001$ by Fisher's exact probability test.

reduced by D-penicillamine treatment ($P<0.001$), but the iron concentration was not.

DISCUSSION

Spontaneous renal cell tumors are uncommon in rats. The incidence of spontaneous tumors (renal cell adenomas and carcinomas) is higher in males than in females. Their incidences in 2-year carcinogenicity studies were reported to be 0.3–0.6% in male and 0.1–0.3% in female F344 rats,^{13–15} 0.1–1.2% in male and 0% in female Sprague-

Dawley rats,^{15, 16} 0–0.4% in male and 0–0.2% in female Wistar rats,^{17, 18} and 0.3% in male and 0% in female Osborne-Mendel rats.¹⁹ In the present study, no male F344 rats developed renal cell tumors, but both sexes of LEC rats developed renal cell tumors at high incidence, with no sex difference in the incidence. The Eker rat, which has a germline mutation in the rat homologue (*Tsc2*) of the human tuberous sclerosis gene,^{20, 21} is a hereditary cancer model and develops multicentric and bilateral renal cell tumors spontaneously at high incidence by 1 year of age.^{22, 23} The incidence and multiplicity of

Table IV. Incidences of Liver and Kidney Lesions in Male LEC Rats Treated with D-Penicillamine

| Treatment | No. of rats | Body weight | Hepatocellular tumor ^{a)} | Kidney | | |
|-------------------------------|-------------|----------------------|------------------------------------|-----------------|--------------------------|------------------|
| | | | | Karyomegaly | Dilated atypical tubules | Renal cell tumor |
| none | 13 | 346±30 ^{b)} | 5 (38%) | 13 (100%) | 5 (38%) | 1 (8%) |
| D-penicillamine ^{c)} | 13 | 361±21 | 1 (8%) | 0 ^{d)} | 0 ^{e)} | 0 |

a) Hepatocellular adenoma plus carcinoma.

b) Mean±SD.

c) Six-week-old male LEC rats were treated with 0.07% D-penicillamine 5 days a week for 56 weeks.

d, e) Significantly different from untreated controls by Fisher's exact probability test: d) $P < 0.001$, e) $P < 0.05$.

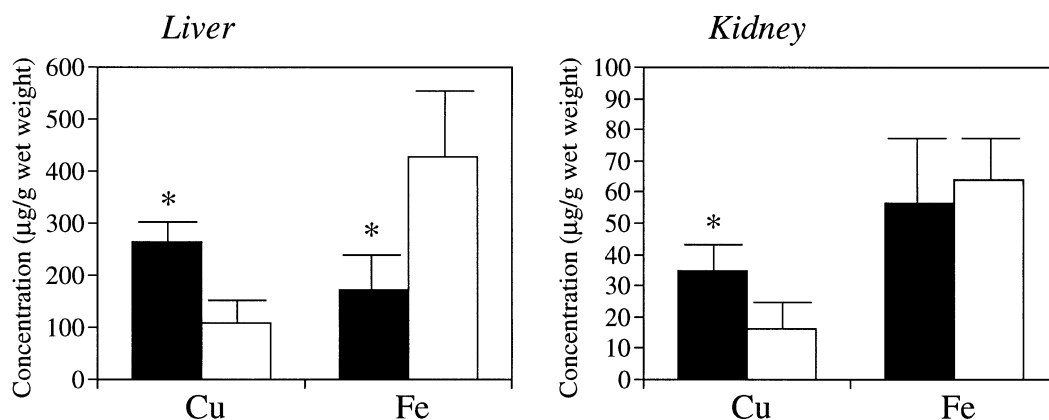


Fig. 5. Copper and iron concentrations in week 56 in the liver and kidneys of LEC rats with and without D-penicillamine treatment ($n=6$). Differences are significant at * $P < 0.001$ by Student's t test. ■ untreated, □ with D-penicillamine.

renal cell tumors in LEC rats are lower than those in Eker rats, and tumors in LEC rats are likely to develop later in life.

Copper and iron are potent generators of reactive oxygen species *in vivo*,²⁴⁾ and these reactive oxygen species induce DNA damage. Both metallothionein-bound and unbound copper increased in the liver and kidneys of LEC rats with jaundice, and the amount of the latter, which plays a crucial role in copper toxicity, is well correlated with the total cytosolic copper content in these organs.²⁵⁾ It has been suggested that hepatic metallothionein-bound copper in LEC rats generates hydroxy-radicals in the presence of hydrogen peroxide in the cells.²⁶⁾ We measured the total copper and iron concentrations in these organs.

The oxidative DNA damage by copper ions includes mutagenesis, strand breaks and 8-OHdG formation.²⁷⁻²⁹⁾ The amount of 8-OHdG in DNA is increased in the liver and kidney of LEC rats,³⁰⁾ and the levels of Cu,Zn-superoxide dismutase, a specific scavenger of superoxide, in LEC rats are lower than those in Wistar rats, especially in the liver and kidney.³¹⁾ Copper and/or hemolysis-induced iron accumulation are considered to play roles in hepato-

carcinogenesis in LEC rats, because copper-induced hemolysis occurs in the period of necrotizing hepatitis, and iron-deficient diet prevents the development of liver tumors.^{32,33)} In the present study, despite the diffuse periportal hemosiderosis of hepatocytes (data not shown) and the increased hepatic iron concentration the incidence of hepatocellular tumors in D-penicillamine-treated LEC rats was lower than that in controls, although we did not measure metal concentrations during the period of jaundice.

Recently, repeated i.p. injections of copper (cupric nitrioltriacetate) were reported to induce renal cell carcinomas in Wistar rats.³⁴⁾ Hemosiderosis caused by copper-induced hemolysis was also suggested to play a role in copper-induced renal carcinogenesis. In the present study, we observed that a significantly higher renal copper concentration and lower iron concentration in LEC rats than in F344 rats continued until at least 2 years old, and long-term D-penicillamine administration inhibited the development of proximal tubule karyomegaly, dilated atypical tubules, and copper, but not iron accumulation in the kidneys. In untreated F344 rats, no renal tumors developed and dilated atypical tubules only rarely developed despite

the increased iron accumulation in the kidneys. Thus, our data suggest that renal tubule accumulation of copper is the major cause of spontaneous renal carcinogenesis in LEC rats.

In our study, D-penicillamine treatment increased the hepatic iron concentration and a similar phenomenon has been observed on trientine treatment.⁸⁾ The reason for this is unknown, but the decrease of hepatic copper concentration by chelating agents may increase intestinal iron absorption or iron uptake into hepatocytes.³⁵⁾

Lesions such as altered tubules³⁶⁾ and atypical tubules and hyperplasia³⁷⁾ are known as renal preneoplastic lesions in rodents. In the present study, dilated atypical tubules were observed in LEC rats, and the incidence of

these lesions increased with age. Dilated atypical tubules are considered to be preneoplastic lesions, rather than reparative hyperplasia after tubular necrosis. Similar lesions have been observed in the kidneys of Eker rats²³⁾ and another strain of rats.³⁷⁾

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