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Effects of D-Galactosamine Hydrochloride and Partial Hepatectomy on Spontaneous Hepatic Injury and Hepatocarcinogenesis in Long-Evans Cinnamon Rats

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To examine the effect of nongenotoxic chemicals on hepatocarcinogenesis in Long-Evans Cinnamon (LEC) rats, we gave 6-week-old male and female LEC rats (*n*=18) weekly subcutaneous injections of D-galactosamine hydrochloride (GalN, 300 mg/kg) in 0.9% NaCl or only 0.9% NaCl for 50 weeks, and killed them in week 62. GalN-treated male rats unexpectedly showed no lethal necrotizing hepatitis. GalN treatment increased the incidence of cholangiofibrosis in males and its severity in females, but did not cause significant increases of hepatocellular tumors in either sex. GalN treatment increased the 5-bromo-2′-deoxyuridine (BrdU)-labeling index of hepatocytes and plasma hepatocyte growth factor, and accelerated megalocytic alterations without reduction of the hepatic copper concentration. Next, male and female LEC rats were subjected to two-thirds partial hepatectomy (PH) or sham hepatectomy in week 8 (*n*=12) or in week 14 (*n*=9), and killed in week 62. PH in week 14 inhibited lethal hepatitis, but PH in week 8 was less effective. PH reduced the hepatic copper concentration to half that of controls. The present data suggest that induction of hepatocyte regeneration by repeated injections of GalN, or by PH just before the onset of jaundice has a significant effect in prevention of hepatic injury of LEC rats, but not enhancement of spontaneous hepatocarcinogenesis.

Key words: D-Galactosamine hydrochloride — Hepatocyte growth factor — Necrotizing hepatitis — Hepatocarcinogenesis — 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*) homologous to the human Wilson's disease gene.[2)] About 20–50% of LEC rats die of necrotizing hepatitis with jaundice 4 to 6 months after birth, and the hepatitis is inherited in an autosomally recessive manner.[3)] Hepatocellular carcinomas develop in rats of 12 months old or more that recover from liver injury.[3–5)]

The oxidative DNA damage by copper ions includes mutagenesis, strand breaks and 8-OHdG formation.[6–8)] The amounts of 8-OHdG in DNA in the liver and kidney of LEC rats are increased.[9)] We and other investigators have observed that liver cell injury in LEC rats is increased by phenobarbital,[10)] clofibrate[10)] or choline-deficient diet[11)] and delayed or inhibited by GalN,[10)] dipyrone,[10)] L-proline,[12)] ascorbic acid,[12)] copper-deficient diet,[12, 13)] D-penicillamine[14)] or trientine.[15)] GalN and dipyrone, an antipyretic drug, are hepatotoxic chemicals, and ascorbic acid is an antioxidant. D-Penicillamine and trientine, copper-chelating agents, inhibit both hepatic injury and liver cancer in LEC rats by reducing the hepatic copper concentration.[14–16)]

GalN has been used for induction of experimental hepatitis in rodents.[17, 18)] It depletes the uridine nucleotide pool and inhibits RNA synthesis.[19)] Its administration to rats causes dose-dependent hepatocellular necrosis and compensatory hepatocyte proliferation. PH also induces rapid hepatocyte proliferation.[20)]

In the present study, we investigated 1) the effect of GalN and PH on spontaneous hepatic injury and hepatocarcinogenesis in LEC rats, and 2) the mechanism by which these treatments prevent lethal hepatitis.

MATERIALS AND METHODS

Animals LEC/Tj rats were bred in the Institute for Animal Experimentation of the University of Tokushima, in specific pathogen-free conditions. F344/DuCrj rats were obtained from Charles River Japan, Inc., Kanagawa. Animals were housed three to a plastic cage with sterilized

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Abbreviations: LEC, Long-Evans Cinnamon; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; GalN, D-galactosamine hydrochloride; BrdU, 5-bromo-2′-deoxyuridine; PH, two-thirds partial hepatectomy; SH, sham hepatectomy; HGF, hepatocyte growth factor.

woodchips for bedding in an air-conditioned room at 23±2°C and 55±10% humidity with a 12 h light/dark cycle, and given pellet diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*.

In our laboratory, the mortality rate of untreated LEC rats during the period of jaundice is 12.4% for males (*n*=193) and 42.3% for females (*n*=52).

Experiment 1 Six-week-old male and female LEC rats (*n*=18) were given weekly subcutaneous injections of 300 mg/kg of GalN (Sigma Chemical Co., St. Louis, MO) dissolved in 0.9% NaCl, or 0.9% NaCl only (2 ml/kg body weight) for 50 weeks. The dose of GalN was chosen from the doses which we have used in previous carcinogenicity studies using F344 rats (unpublished data). Animals were examined daily for the emergence of jaundice and their body weight was recorded once a week. All surviving rats were killed under ether anesthesia in week 62 and all organs including all hepatic lobes were fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin, and examined histologically. Histological types of liver tumors were classified as reported.[21]

Experiment 2 Six-week-old male LEC rats were given weekly subcutaneous injections of GalN (300 mg/kg) in 0.9% NaCl or 0.9% NaCl only for 5, 12 or 25 weeks (*n*=5 at each point). They were killed 7 days after the final injection, and the copper concentration of their liver was measured.

Experiment 3 The labeling index with BrdU (Sigma Chemical Co.) of hepatocytes after GalN treatment was measured. Six-week-old male LEC rats (*n*=5 at each point) were given weekly subcutaneous injections of GalN (750 mg/kg or 300 mg/kg) in 0.9% NaCl or 0.9% NaCl only for three weeks. On days 0, 2, 3 and 7 after the final injection, rats were given an intraperitoneal injection of 50 mg/kg of BrdU and killed 30 min later. The liver was fixed in 95% ethanol and embedded in paraffin. Sections were stained with anti-BrdU antibody (Becton Dickinson Immunocytometry Systems, Mountain View, CA) using a DAKO LSAB kit, peroxidase (DAKO Co., Carpinteria, CA) after partial denaturation of double-stranded DNA. Livers were also fixed in buffered formalin and examined histologically.

Experiment 4 Six-week-old male LEC rats were given weekly subcutaneous injections of GalN (300 mg/kg) in 0.9% NaCl or 0.9% NaCl only for three weeks (*n*=4 at each point). Rats were killed on day 0, 1, 2 or 3 after the final injection, and their plasma HGF concentration was measured by sandwich enzyme-linked immunosorbent assay (rat HGF-EIA kit, Institute of Immunology, Tokyo). Frozen sections of the liver were fixed in ice-cold acetone for 10 min and stained with polyclonal anti-rat HGF antibody using a DAKO LSAB kit.

Experiment 5 Male and female LEC rats were subjected to PH[20] or SH at 8 weeks (*n*=12) or 14 weeks (*n*=9). Rats were killed in week 62 and examined histologically.

Experiment 6 Ten-week-old male LEC and F344 rats were subjected to PH or SH and killed on day 0, 3, 7 or 14 (*n*=3 at each point). The liver/body weight and hepatic copper concentration were measured. The liver was examined histologically.

Analysis of copper concentration Samples of the liver (0.5 g) were stored at −80°C until use. They were ashed with nitric acid, and their copper concentrations were measured in an atomic absorption spectrophotometer (AA-782, Nippon Jarrel Ash, Co., Kyoto).

Statistical analyses Data on relative liver weights, the numbers of tumors and BrdU labeling indices were ana-

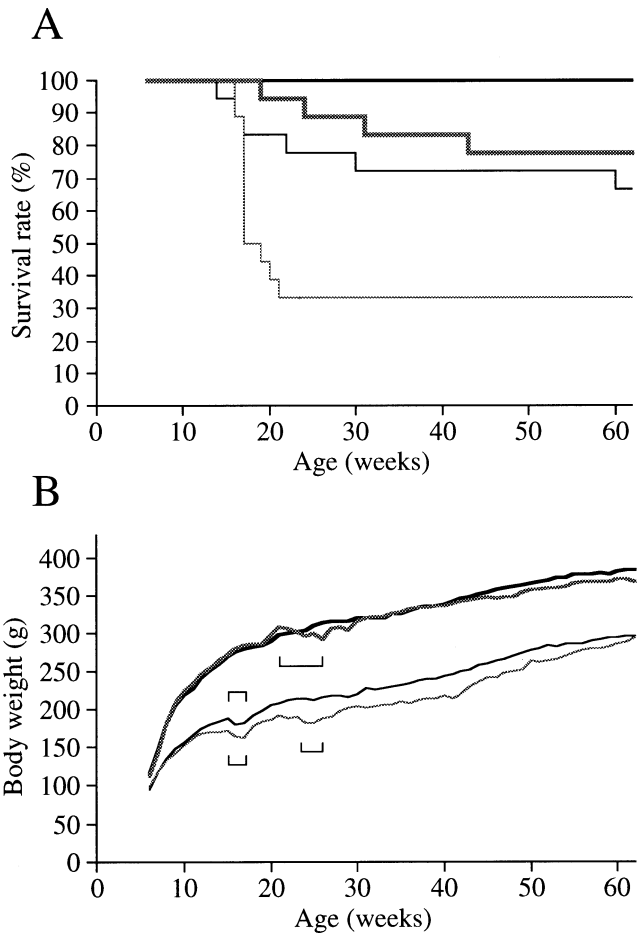

Fig. 1. Survival rates (A) and growth curves (B) of male and female LEC rats given weekly subcutaneous injections of 300 mg/kg of GalN in 0.9% NaCl or only 0.9% NaCl for 50 weeks (Experiment 1). Surviving rats were killed in week 62 after a 6-week recovery period. —— GalN, males; —— 0.9% NaCl, males; —— GalN, females; —— 0.9% NaCl, females; ⌊⌋ inhibition of body weight gain.

Table I. Effect of GalN on Carcinogenesis of LEC Rats (Experiment 1)

Sex	Treatment	Initial no. of rats	Jaundice	Effective no. of rats[a)]	Liver					Other tumors
					Liver/body weight (%)	No. of tumors/rat (≥5 mm)	Hepatocellular adenoma	Hepatocellular carcinoma	Cholangio-fibrosis	
M	GalN	18	3 (17%)[b)]	18	5.6±3.2[d, e)]	1.4±1.8	12 (67%)	1 (6%)	17 (94%)[c)]	0
	0.9% NaCl	18	13 (72%)	14	3.3±0.7	0.5±0.6	7 (50%)	0	4 (29%)	2[f)]
F	GalN	18	17 (94%)	13	9.5±3.6	0.5±0.8	4 (31%)	1 (8%)	13 (100%)	0
	0.9% NaCl	18	18 (100%)	6	8.1±2.2	0.5±0.5	3 (50%)	0	6 (100%)	0

a) ≥60 weeks old.
b, *c*) Significantly different from the 0.9% NaCl group at [b)]$P<0.01$ and [c)]$P<0.001$ (Fisher's exact probability test).
d) Mean±SD.
e) Significantly different from the 0.9% NaCl group at $P<0.02$ (Student's *t* test).
f) One renal cell carcinoma and one thymic carcinoma.

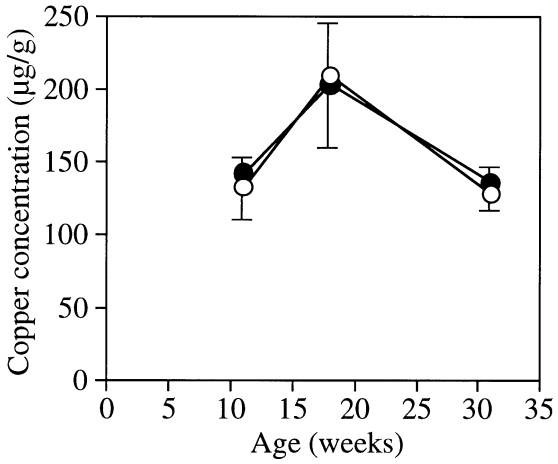

Fig. 2. Hepatic copper concentrations of male LEC rats given weekly subcutaneous injections of 300 mg/kg of GalN in 0.9% NaCl or only 0.9% NaCl for 5, 12, or 25 weeks (Experiment 2). Rats were killed 7 days after the final injection. ● GalN, ○ 0.9% NaCl. Bars, SD.

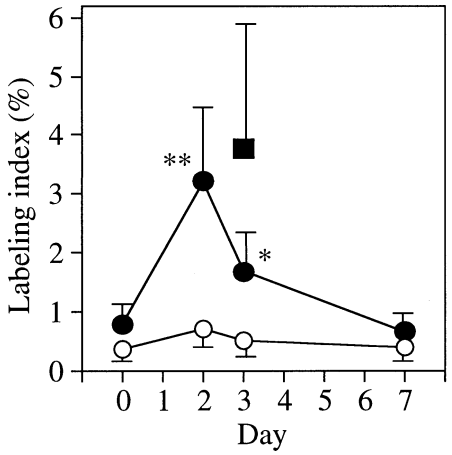

Fig. 3. BrdU-labeling indices of hepatocytes of male LEC rats (n=5) after treatment with 750 or 300 mg/kg of GalN in 0.9% NaCl or only 0.9% NaCl for three weeks (Experiment 3). Rats were killed on days 0, 2, 3 and 7. ** $P<0.01$, * $P<0.05$ (Student's *t* test). ■ GalN, 750 mg/kg; ● GalN, 300 mg/kg; ○ 0.9% NaCl. Bars, SD.

lyzed by using Student's *t* test and the incidences of hepatic lesions were analyzed by using Fisher's exact probability test.

RESULTS

Long-term test on GalN In Experiment 1, the incidence of jaundice in GalN-treated rats was higher than that in control rats. None of the GalN-treated male rats died, but three control male rats died of necrotizing hepatitis between week 19 and 31, five GalN-treated female rats between week 14 and 30, and 12 control female rats between week 16 and 21 (Fig. 1A). One 0.9% NaCl-treated male rat died of cecal ulcer in week 43, and one GalN-treated female rat died of hepatocellular carcinoma with lung metastasis in week 60. The survival rate of males was higher than that of females, and the rates in GalN-treated rats of both sexes were higher than those of the controls. Inhibition of body weight gain of rats with jaundice was seen twice in control female rats and once in control males and GalN-treated females, but not in GalN-treated males (Fig. 1B). The average number of gross hepatocellular tumors (≥5 mm in diameter) in GalN-treated males was higher than that in control males, the difference not being significant (Table I). Hepatocellular carcinoma developed at low incidence in only GalN-treated rats. The liver/body weight ratio was higher in GalN-treated animals of both sexes because of cholangiofibrosis, and its incidence in GalN-treated males was higher than that in control males ($P<0.001$).

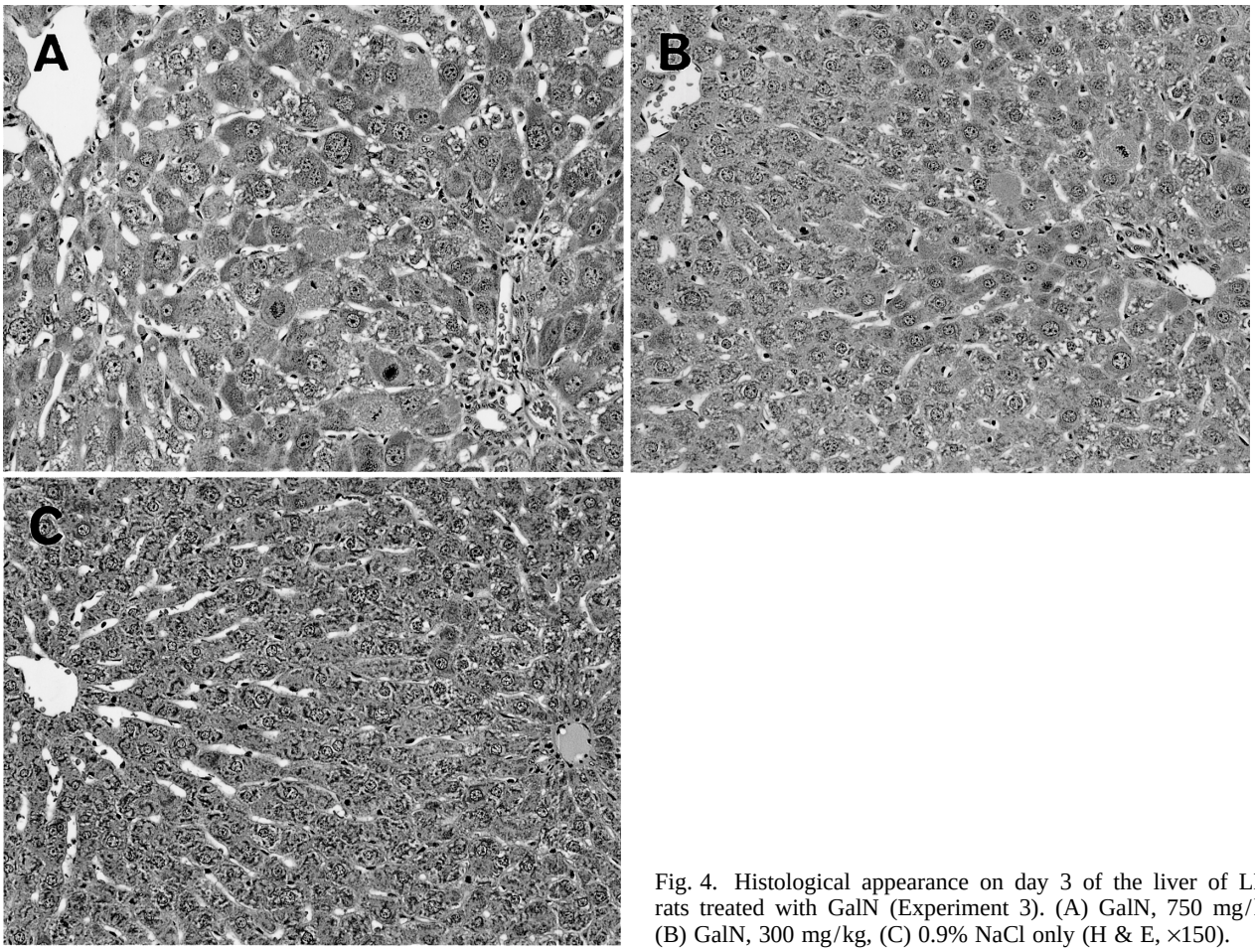

Fig. 4. Histological appearance on day 3 of the liver of LEC rats treated with GalN (Experiment 3). (A) GalN, 750 mg/kg, (B) GalN, 300 mg/kg, (C) 0.9% NaCl only (H & E, ×150).

Hepatic copper concentrations of GalN-treated rats In Experiment 2, the average copper concentrations in the liver at all times were similar in GalN-treated and control rats (Fig. 2). Serum aspartate aminotransferase and alanine aminotransferase measured with an automatic analyzer were similar in the two groups (data not shown).

Effect of GalN on cell proliferation In Experiment 3, the BrdU labeling index of hepatocytes in rats treated with 300 mg/kg of GalN was 4.6 times that in the controls on day 2 ($P<0.01$), and decreased to close to the control level on day 7 (Fig. 3). Histologically, liver cell enlargement with large nuclei, which is always observed in LEC rats recovering from jaundice, was observed in GalN-treated rats, especially in the 750 mg/kg-treated group, on day 3 (Fig. 4).

Plasma HGF concentration and immunohistochemistry of the liver in GalN-treated rats In Experiment 4, the plasma HGF increased on days 1 and 2 and decreased to near the normal level on day 2 except in one rat (Fig. 5).

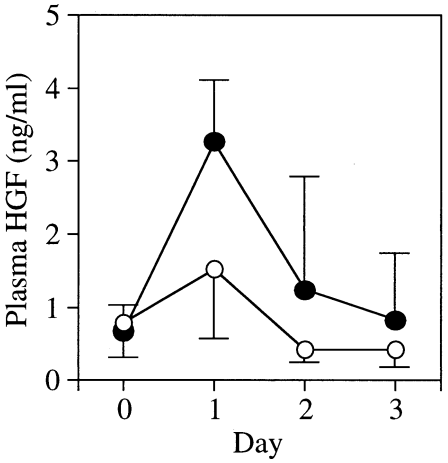

Fig. 5. Plasma HGF of male LEC rats (n=4) after treatment with 300 mg/kg of GalN in 0.9% NaCl or only 0.9% NaCl for three weeks (Experiment 4). Rats were killed on days 0, 1, 2 and 3. ● GalN, ○ 0.9% NaCl. Bars, SD.

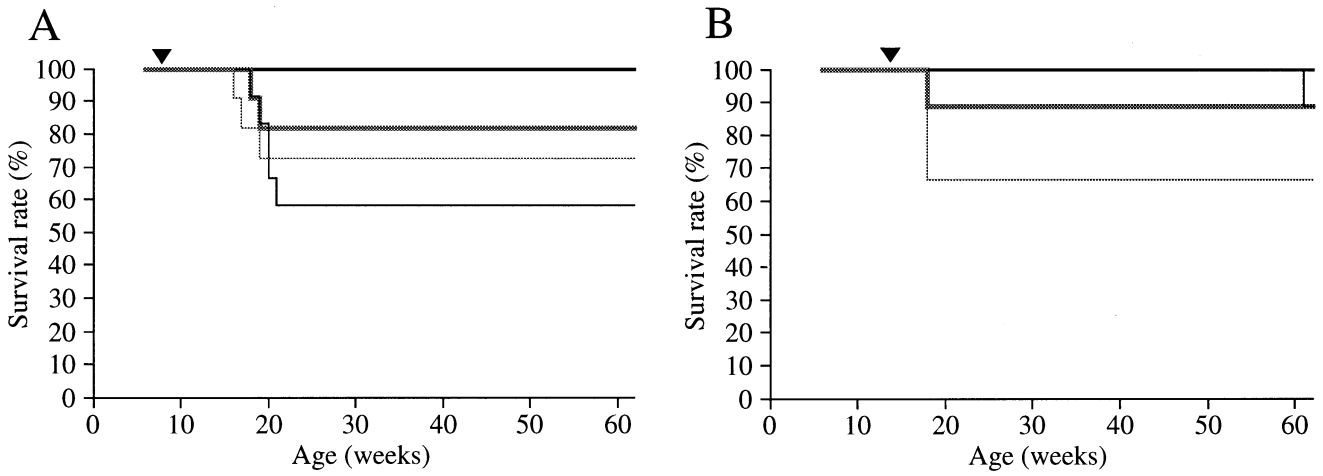

Fig. 6. Survival rates of male and female LEC rats given PH or SH. Rats were subjected to PH or SH in week 8 (A) or week 14 (B), and killed in week 62 (Experiment 5). — PH, males; — SH, males; — PH, females; — SH, females; ▼ PH or SH.

Table II. Effect of PH in Week 8 or 14 on Carcinogenesis in LEC Rats (Experiment 5)

Sex	Treatment	Initial no. of rats	Jaundice	Effective no. of rats[a)]	Liver						Other tumors
					Liver/body weight (%)	No. of tumors/rat (≥5 mm)	Hepato-cellular adenoma	Hepato-cellular carcinoma	Cholangio-cellular carcinoma	Cholangio-fibrosis	
PH or SH at 8 weeks											
M	PH	12	3 (25%)	12	3.2±0.4[b, c)]	1.3±1.4	7 (58%)	0	0	0	0
	SH	11	7 (64%)	9	3.6±0.4	1.7±1.4	7 (78%)	0	0	2 (22%)	1[e)]
F	PH	12	12 (100%)	7	4.3±0.4[d)]	0.7±1.0	3 (43%)	1 (14%)	0	7 (100%)	0
	SH	11	11 (100%)	8	8.0±2.4	0.4±0.7	2 (25%)	0	0	8 (100%)	1[f)]
PH or SH at 14 weeks											
M	PH	9	3 (33%)	9	3.4±0.6	1.1±1.1	5 (56%)	1 (11%)	1 (11%)	0	1[g)]
	SH	9	7 (78%)	8	3.4±0.5	0.4±0.5	3 (38%)	0	0	1 (13%)	0
F	PH	9	8 (89%)	9	5.0±1.4[d)]	1.0±0.9	5 (56%)	1 (11%)	0	7 (78%)	0
	SH	9	9 (100%)	6	8.2±1.6	0.6±0.5	3 (50%)	0	0	5 (83%)	0

a) ≥60 weeks old.
b) Mean±SD.
c, *d*) Significantly different from the SH group at [c)]$P<0.05$ and [d)]$P<0.01$ (Student's *t* test).
e) One renal cell adenoma.
f) One renal cell carcinoma.
g) One malignant lymphoma.

Immunohistochemically, slight increases of the number of HGF-positive mesenchymal cells were observed in GalN-treated rats on day 2.

Effect of PH on spontaneous liver injury and hepatocarcinogenesis In Experiment 5, PH completely prevented death in the period of jaundice in males at both 8 and 14 weeks (Fig. 6). PH increased the survival rate of females of 14 weeks, but not 8 weeks. PH decreased the liver/body weight ratio by preventing severe cholangiofibrosis in females of both 8 and 14 weeks, although the incidences of cholangiofibrosis were similar in these groups (Table II). Hepatocellular and cholangiocellular carcinomas developed at low incidences in only PH groups of both sexes.

Hepatic copper concentrations of hepatectomized rats In Experiment 6, the liver/body weight ratios in hepatectomized F344 rats recovered to 97% of that of sham-operated F344 rats on day 14, but that of LEC rats was 86%

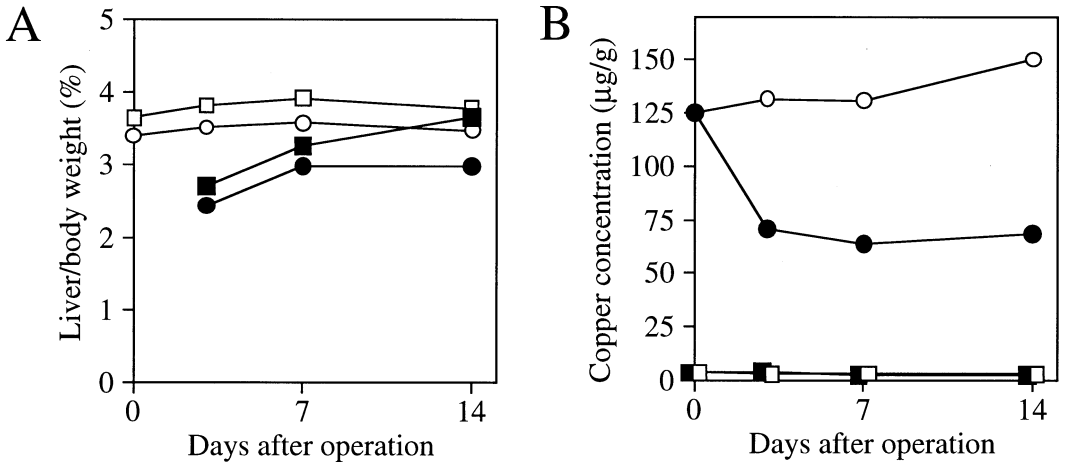

Fig. 7. Liver/body weight (A) and hepatic copper concentration (B) of 10-week-old male LEC and F344 rats (n=3) after PH or SH (Experiment 6). Rats were killed on days 0, 3, 7 and 14. ● LEC, PH; ○ LEC, SH; ■ F344, PH; □ F344, SH.

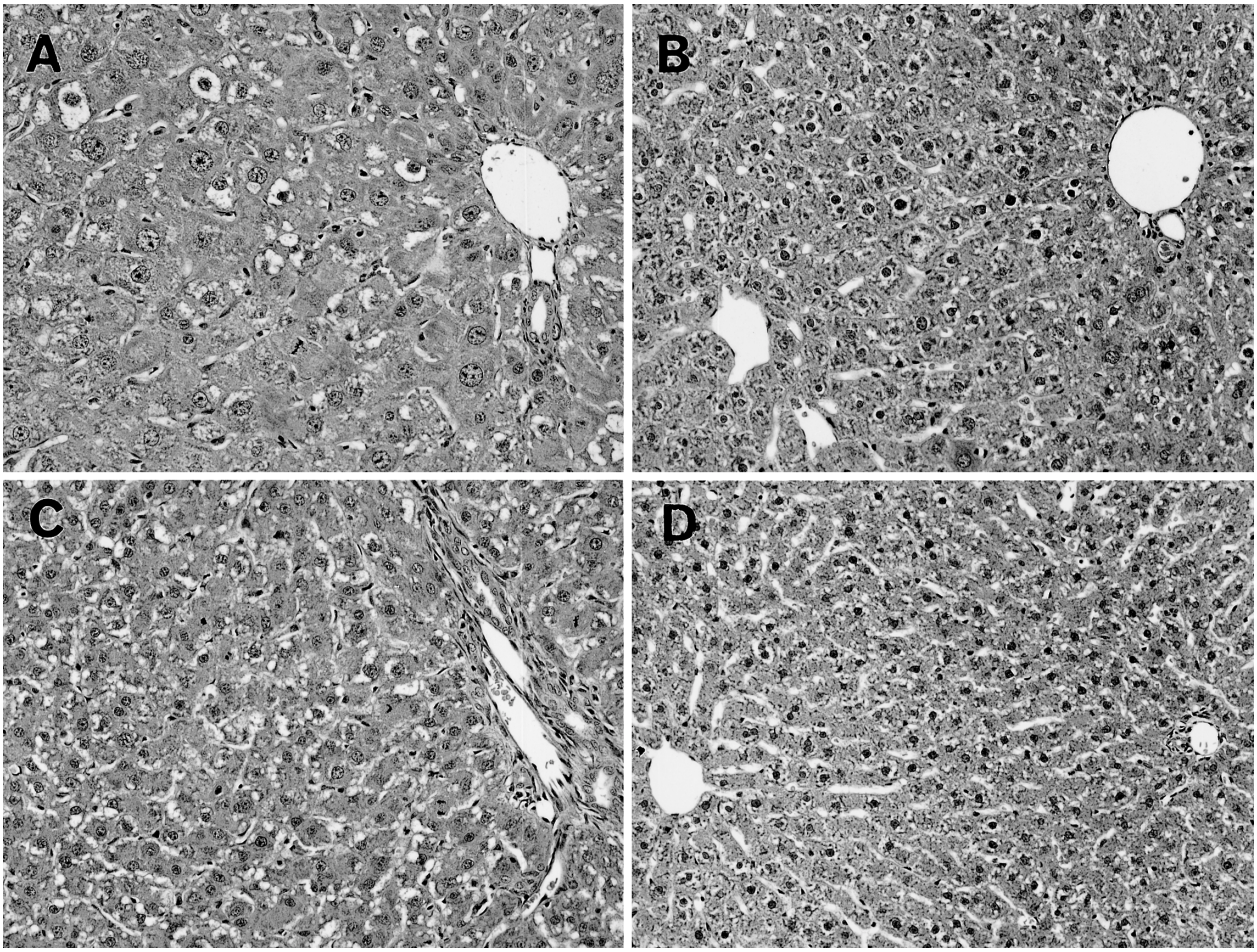

Fig. 8. Histological appearances on day 3 of the liver of hepatectomized and sham-operated rats (Experiment 6). (A) LEC rat, PH, (B) LEC rat, SH, (C) F344 rat, PH, (D) F344 rat, SH (H & E, ×150).

of the control level (Fig. 7). In LEC rats, the copper concentration of hepatectomized rats was 45% of that of control rats on day 14. Histologically, nuclear enlargement in hepatectomized LEC rats was more prominent than that in hepatectomized F344 rats (Fig. 8).

DISCUSSION

Excess copper accumulation in hepatocytes induces necrotizing hepatitis with jaundice in LEC rats. Necrotizing hepatitis is reported to be more severe in females than in males,[22] as also shown in the present study. Although the severity of hepatic injury is related to the hepatic copper concentration, it is unknown why lethal hepatitis develops 4 to 6 months after birth. In the present study, we demonstrated that 1) both long-term administration of GalN and PH in LEC rats of 8 or 14 weeks old inhibited the emergence of jaundice and increased the survival rates, 2) both GalN and PH induced hepatocyte proliferation, 3) PH reduced the hepatic copper concentration for at least two weeks, but GalN did not reduce it. These results suggest that hepatocyte regeneration induced by GalN or PH plays a more significant role than decrease of the hepatic copper concentration in preventing severe hepatic injury. In a recent study, we observed that short-term administration of a necrogenic dose of *N*-diethylnitrosamine, a potent hepatocarcinogen, also reduced the mortality of LEC rats (unpublished data).

HGF is a potent mitogen and a cytoprotective agent for hepatocytes. There are reports that GalN increases HGF mRNA in the liver of rats,[23] and that HGF works as an anti-hepatitis agent against GalN-induced liver injury of HGF transgenic mice.[24] In the present study, GalN treatment increased the plasma HGF on day 1. There are also reports that in LEC rats 1) plasma HGF, HGF mRNA expression and HGF-positive non-parenchymal cells in the liver increase during the phase of fulminant hepatitis, and decrease during the chronic phase, and 2) HGF-positive cells increase in the liver 24 h after PH.[25] These findings also support the idea that HGF production by GalN treatment may be related to the low mortality rate of LEC rats in the present study.

Characteristic histological findings on hepatocytes of LEC rats after the period of jaundice were megalocytic alteration and cholangiofibrosis,[3] the latter being more severe in females than in males. Megalocytic hepatocytes are polyploid cells induced by intracytoplasmic copper accumulation, and the DNA content of these cells reaches 64n.[10, 26] Proliferation of megalocytic hepatocytes is low, and most of these cells were not labeled on administration of BrdU for one week with a mini-osmotic pump (unpublished data). GalN treatment induced earlier development of megalocytic alteration of hepatocytes without reduction of hepatic copper accumulation than 0.9% NaCl treatment in the present study. Thus, megalocytic alteration may occur as a protective reaction against the toxicity of GalN as well as the process of hepatocyte regeneration.

Cholangiofibrosis is induced by various hepatocarcinogens and hepatotoxic agents including GalN.[27–29] Long-term administration of copper-chelating agents reduces copper and 8-OHdG in the liver, and inhibits cholangiofibrosis in LEC rats.[15, 16] In the present study, cholangiofibrosis was enhanced by GalN without reduction in hepatic copper accumulation, and inhibited by PH, which reduced hepatic copper accumulation. The hydroxy radical and/or another fibrogenic factor(s) may be related to the development of cholangiofibrosis.

Cell proliferation is an important factor in the carcinogenicities of nongenotoxic compounds,[30] but toxic agents are not necessarily carcinogenic in two-year carcinogenicity bioassays.[31, 32] Recently, using male F344 rats, we found that hepatocellular carcinomas were not induced by repeated injections of GalN alone for more than 70 weeks, but that after initiation with *N*-diethylnitrosamine, GalN enhanced hepatocarcinogenesis (unpublished data). At first, we supposed that GalN would enhance hepatocarcinogenesis in LEC rats, because liver tumors develop spontaneously in this strain of rats, and it is suspected that LEC rats have a cancer susceptible gene(s).[33] However, in the present study we could not show that repeated injections of GalN without an initiator enhanced hepatocarcinogenesis. Therefore, in these rats predisposing conditions, such as a cancer susceptibility gene(s) and copper accumulation, may be insufficient for GalN-induced tumor promotion, or a longer observation period may be necessary to induce liver cancer because in this study hepatocellular carcinomas developed at low incidence in GalN-treated and hepatectomized rats, but not in the respective controls.

This study showed that GalN and PH induced early development of megalocytic alteration, and GalN enhanced cholangiofibrosis in LEC rats. Both GalN and PH reduced the severity of necrotizing hepatitis, but did not enhance hepatocarcinogenesis under the present experimental conditions. These findings suggest that hepatocyte regeneration and/or reduction of hepatic copper accumulation are significant in preventing lethal hepatitis in LEC rats.

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