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Preventive effects of the deleted form of hepatocyte growth factor against various liver injuries

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Abstract

The effects of a naturally occurring deleted form of hepatocyte growth factor (HGF) on hepatic disorder were studied in various models of hepatic failure. The pretreatment of rats and mice with the deleted form of HGF prevented the liver injuries and coagulopathy induced by endotoxin, dimethylnitrosamine and acetaminophen and reduced the mortality due to hepatic dysfunction induced by these hepatotoxins. The concurrent administration of the deleted form of HGF also prevented the liver injury and hepatic fibrosis in mice treated with α-naphthylisothiocyanate and in rats treated with dimethylnitrosamine. Moreover, the deleted form of HGF normalized the results of the bromosulphalein-clearance test and ameliorated jaundice in rats with periportal cholangiolitic hepatopathy induced by α-naphthylisothiocyanate. The deleted form of HGF also reversed the coagulopathy in rats with hepatic disorder induced by dimethylnitrosamine or by 70% resection of cirrhotic liver (induced by carbon tetrachloride). In Long–Evans cinnamon rats receiving vehicle, 20 out of 21 animals died within 4 days after the onset of jaundice. After infusion of the deleted form of HGF for 4 days, 7 out of 20 Long–Evans cinnamon rats survived. These results indicate that the deleted form of HGF could have therapeutic potency in patients with severe hepatic failure. © 1998 Elsevier Science B.V.

Keywords: HGF (hepatocyte growth factor); Hepatic disorder; Coagulopathy; Hepaplastin test; Endotoxin

1. Introduction

Patients with terminal liver disease frequently have various combined symptoms associated with severe hepatic dysfunction (Atillasoy and Berk, 1995; Caraceni and Van Thiei, 1995). However, the pathogenesis of terminal-stage liver disease and effective therapeutics for the patients are not still established.

Hepatocyte growth factor (HGF) was initially isolated from rat platelets (Nakamura et al., 1986) and thereafter from the plasma of patients with fulminant hepatic failure (Gohda et al., 1988). A major variant of HGF, the deleted form of HGF, has been purified from the conditioned medium of human fibroblasts (Higashio et al., 1990). The deleted form of HGF is a heparin-binding basic protein with an apparent molecular mass of 80 kDa and is a heterodimer composed of a large α-chain with a apparent molecular mass of 52–56 kDa and a small β-chain with a apparent molecular mass of 30–34 kDa as well as HGF. The deleted form of HGF cDNA, which lacks 15 nucleotides encoding a 5-amino acid residue in the first kringle domain of HGF, has been isolated from the human fibroblast cDNA library (Shima et al., 1991a). The deleted form of HGF is different from HGF in its tertiary structure because the deleted form of HGF-specific antibodies recognize three-dimensional structures newly formed in the protein moiety by the deletion of 5 amino acids (Shima et al., 1994). Matsumoto et al. (1991) have reported that the deleted form of HGF is 1.4-fold more potent than HGF in stimulating DNA synthesis in adult rat hepatocytes in primary culture. It is recognized that the deleted form of HGF and HGF are distinguishable in their target cell specificity and stimulation of growth as follows (Shima et al., 1994). Dose–response curves for the stimulation of DNA synthesis in rat hepatocytes by the deleted form of HGF and HGF are very similar up to about 10 ng/ml, but are significantly different at higher concentrations. HGF activity is markedly decrease over a dose range of 10 to 500 ng/ml, while the deleted form of HGF has maximal

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activity over the same dose range. The specific activity of the deleted form of HGF is maximally 1.9-fold higher than that of HGF in this dose range. In addition to the difference between the deleted form of HGF and HGF in the stimulation of hepatocytes, the deleted form of HGF is less potent (1/10–1/20) than HGF in the stimulation of DNA synthesis in mesenchymal cells such as human umbilical vein endothelial cells and human aorta smooth muscle cells. These results indicate that the deleted form of HGF may be more specific for the growth of hepatocytes than HGF (Shima et al., 1994).

Since plasma levels of the deleted form of HGF and HGF in patients with liver diseases are higher than those of normal subjects, these growth factors appear to act as the effecter molecules responsible for repairing injured liver tissue (Shima et al., 1991b; Tsubouchi et al., 1991). However, there is no evidence that the deleted form of HGF is useful for treating the principal complication of severe liver diseases. Recently, we showed that the deleted form of HGF ameliorates disordered hepatic protein synthesis in models of liver failure (Masunaga et al., 1996). A systematic investigation should be made to evaluate the possibility of therapeutically using the deleted form of HGF for hepatic disorders generated by various causes. In this study, we examined whether the treatment with the deleted form of HGF could reduce mortality of animals with severe hepatic failure induced by the administration of a lethal dose of several hepatotoxins, ameliorate clinico-chemically hepatic dysfunction and prevent morphological liver injury and hepatic fibrosis in models of liver failure.

2. Materials and methods

2.1. Materials

The deleted form of HGF purified from the culture broth of Namalwa cells transfected with expression vector comprising the human deleted form of HGF cDNA was used (Shima et al., 1991a). The deleted form of HGF was diluted with sterile phosphate buffered saline containing 0.01% Tween 80. Control animals received the same volume of the vehicle.

2.2. Animals

ICR mice (male, 7 weeks old, 28–30 g), C.B-17/Icr-scid mice (female, 7 weeks old, 18–20 g), Wistar rats (male, 7 weeks old, 180–200 g), Long–Evans cinnamon rats (female, 13 weeks old), which were used as a model of spontaneous fulminant hepatic failure and Long–Evans rats (female, 13 weeks old), which were normal controls for Long–Evans cinnamon rats, were used in this study. The animals were maintained on standard chow and water ad libitum. They were housed under controlled temperature (23 ± 1°C), humidity (55 ± 5%) and lighting (7.00 a.m. to 7.00 p.m.).

2.3. Endotoxin-induced hepatic failure

Wistar rats ($n = 10$) were given the deleted form of HGF (0.5 mg/kg i.v. twice daily for 7 days) from just after the injection of *Propionibacterium acnes* (*P. acnes*, 30 mg/kg i.v.). On day 7, the rats were killed 3 h after the injection of *S. typhosa* endotoxin (300 μg/kg i.v.) to determine plasma clotting time, levels of fibrinogen and γ-glutamyl transpeptidase.

The effect of the deleted form of HGF on the mortality of mice with severe hepatic failure induced with endotoxin was investigated. ICR mice ($n = 25$) were given *P. acnes* (30 mg/kg i.v.) on day 9 before an injection of *S. typhosa* endotoxin (100 μg/kg i.v). The deleted form of HGF (3 mg/kg i.v. twice daily) was given to the mice for 4 days before the injection of *S. typhosa* endotoxin. Similarly, the deleted form of HGF was given at the same dose to ICR mice ($n = 25$) for 4 days before the i.v. injection of galactosamine (500 mg/kg) plus *E. coli* endotoxin (10 mg/kg) or the i.v. injection of a lethal dose (20 mg/kg) of *E. coli* endotoxin.

2.4. Hepatotoxin-induced hepatic failure

ICR mice ($n = 5$) received the deleted form of HGF (0.3, 1.5 and 7.5 mg/kg i.v. twice daily) for 2 days before the administration of acetaminophen (800 mg/kg p.o.) or dimethylnitrosamine (15 μl/kg i.v.). The mice were killed 6 or 24 h after the administration of acetaminophen or dimethylnitrosamine to determine plasma clotting time, serum level of aspartate aminotransferase, hepatic glutathione (GSH) content and hepatic superoxide dismutase activity. The liver tissue of mice 24 h after the injection of dimethylnitrosamine was stained with hematoxylin–eosin and was subjected to morphological examination. Next, the mortality of ICR mice ($n = 25$) receiving the deleted form of HGF (3 mg/kg i.v. twice daily) for 4 days before the administration of a lethal dose of acetaminophen (800 mg/kg p.o.) or dimethylnitrosamine (15 μl/kg i.v.) was examined.

In addition, the deleted form of HGF (0.15 and 0.5 mg/kg i.v. twice daily) was given to Wistar rats from just after the induction of periportal cholangiolitic hepatopathy by α-naphthylisothiocyanate (100 mg/kg p.o.) for 3 days until 12 h before the rats were killed. These rats were killed to determine the value (%) of bromosulphalein clearance test and the serum levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase, total bilirubin and free-cholesterol. For examination of bromosulphalein clearance, the reagent of Daiichi Hepatosulfalein (bromosulphalein 50 mg/kg i.v., Tokyo, Japan) was given to the rats 30 min before blood sampling.

2.5. Liver damage and fibrosis induced by repeated administration of hepatotoxins

In the following examination, C.B-17/Icr-scid mice were used to avoid immunological response to human protein, the deleted form of HGF, during a long-term administration (Bosma et al., 1983). α-naphthylisothiocyanate (50 mg/kg) was orally administered to mice twice a week for 12 weeks. These mice received the deleted form of HGF (2.5 mg/kg i.v.) 5 times a week for 12 weeks from the beginning of the experiment. The mice were killed the day after the last injection of the deleted form of HGF. Serum levels of aspartate aminotransferase, alanine aminotransferase, glucose, free-cholesterol and hepatic hydroxyproline content were measured. The liver tissue was stained by silver impregnation and subjected to morphological examination.

Wistar rats received dimethylnitrosamine (10 μl/kg i.p.) weekly on the first three consecutive days for 4 weeks, according to the method of Jézéquel et al. (1987). The deleted form of HGF (0.05 or 0.5 mg/kg i.v. twice daily) was given to the rats ($n = 10$) from the beginning of the experiment up to day 28. The rats were killed on day 29 and the liver tissue was stained by silver impregnation and subjected to morphological examination. Plasma clotting time, platelet counts, serum levels of aspartate aminotransferase, alanine aminotransferase, total bilirubin, glucose and free-cholesterol and the hepatic content of GSH, glycogen and hydroxyproline were determined. To evaluate the therapeutic usefulness of the deleted form of HGF for patients with liver cirrhosis, the deleted form of HGF (0.5 mg/kg i.v. twice daily) was given to rats ($n = 10$) with established cirrhosis for a week. The rats were killed the day after the last injection of the deleted form of HGF. Plasma clotting time, fibrinogen, serum levels of total protein, albumin, high-density lipoprotein (HDL)-cholesterol, aspartate aminotransferase, alanine aminotransferase and total bilirubin and liver weight were measured.

2.6. Coagulopathy in an acute hepatic failure

The effect of the deleted form of HGF on coagulopathy in acute hepatic failure was studied by using rats with 70% partial resection of normal or cirrhotic liver. A model of acute-on-chronic hepatic failure was made by performing 70% partial hepatectomy of cirrhotic liver. Cirrhosis was induced by the repeated administration of carbon tetrachloride (CCl_4) (1 ml/kg p.o. twice a week for 12 weeks). The partial hepatectomy of normal and cirrhotic liver was performed by excising the medial and left hepatic lobes under ether anesthesia (Higgins and Anderson, 1931). The deleted form of HGF (0.1 and 0.3 mg/kg/day) was constantly infused into the rats for 48 h, from just after hepatectomy, through a catheter placed in the jugular vein by means of an infusion pump (Eicom, Japan). The rats were killed immediately after the infusion of the deleted form of HGF. Plasma clotting time, serum levels of total protein and HDL-cholesterol, hepatic GSH level and liver weight were measured.

2.7. A spontaneous fulminant hepatic failure model Long–Evans cinnamon rats

The deleted form of HGF (1 mg/kg/day) or vehicle was constantly infused to Long–Evans cinnamon rats through a catheter placed in the jugular vein by using an infusion pump. The infusion was started just after the onset of jaundice and bilirubinuria. The number of survivors in the group ($n = 20$) given the deleted form of HGF for 4 days was compared with that in the group ($n = 21$) given vehicle. In the survivors, plasma clotting time, serum levels of alanine aminotransferase and aspartate aminotransferase were determined and the liver tissue was subjected to copper-staining and then to morphological examination.

2.8. Sampling and analysis

Blood was collected, using a vacuum tube (Venoject, Terumo, Tokyo, Japan) or syringe, from the inferior caval vein of rats or mice under ether anesthesia. A vacuum tube containing sodium citrate solution (3.8%) or without anticoagulant was used for the sampling of plasma or serum. Plasma clotting time was assayed with reagents of the Eisai Hepaplastintest kit (Tokyo, Japan) and an Amelung KC-40 coagulometer (Lemgo, Germany) and was expressed as hepaplastin test time (s). The fibrinogen level was assayed with reagents of Eisai Fibrinogen kit (Tokyo, Japan) and a KC-40 coagulometer. Serum levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin, γ-glutamyl transpeptidase, total protein, glucose, free- and HDL-cholesterol were determined with a Hitachi type 7150 automatic analyzer (Tokyo, Japan), using commercial test reagents (Daiichi Pure Chemicals, Tokyo, Japan). After the liver was excised for weighing, a piece of liver tissue was homogenized in cold saline and used to measure the levels of glycogen (Passonneau and Lauderdale, 1974), GSH (Saville, 1958; Gaitonde, 1967), superoxide dismutase activity (Beauchamp and Fridovich, 1971) and protein (by using a Bio-Rad protein assay kit). The hepatic collagen content was measured as the hydroxyproline content. The hydroxyproline content was measured after hydrolysis of livers in 6 M HCl at 120°C for 16 h by the method of Kivirikko et al. (1967). Liver tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections were stained with hematoxylin–eosin for routine examination, by reticulin silver impregnation for the demonstration of collagen and by Timm's method for copper.

Table 1
Effects of dHGF (0.5 mg/kg) on hepaplastin test time, levels of fibrinogen and γ-glutamyl transpeptidase (γ-GTP) in rats with fulminant hepatitis induced by *P. acnes* + *S. typhosa* endotoxin (LPS)

		Hepaplastin (s)	Fibrinogen (g/l)	γ-GTP (U/l)
Normal		30.9 ± 1.6	1.98 ± 0.03	2.3 ± 0.1
P. acnes +	vehicle	48.7 ± 4.8	0.38 ± 0.11	7.0 ± 0.8
S. typhosa LPS	dHGF	35.6 ± 1.5[b]	0.55 ± 0.11	5.2 ± 0.5[a]

Each value represents the mean ± S.E.M. for 9–10 rats.
[a] $P < 0.05$, [b] $P < 0.01$ significant differences from control group receiving vehicle.

Table 2
Effects of dHGF on mortality following the administration of various hepatotoxins in mice

		Day							
		0	1	2	3	4	5	6	7
P. acnes +	vehicle	25	0	0	0	0	0	0	0
S. typhosa endotoxin	dHGF	25	12	10	10	10	10	10	10
Galactosamine +	vehicle	25	9	7	6	5	5	5	5
E. coli endotoxin	dHGF	25	23	21	19	19	19	19	19
E. coli endotoxin	vehicle	25	25	9	4	3	3	3	3
	dHGF	25	25	17	15	15	14	14	14
Dimethylnitrosamine	vehicle	25	25	7	3	0	0	0	0
	dHGF	25	25	25	25	25	25	25	25
Acetaminophen	vehicle	25	8	8	8	8	8	8	8
	dHGF	25	25	25	25	25	25	25	25

Each value represents the number of mice that survived.

2.9. Statistical analysis

The results are expressed as the mean ± S.E. The data were analyzed by analysis of variance. Differences between groups were analyzed by using the multiple comparison procedure of Fisher's protected least significant difference test. Significance was established at $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Endotoxin induced hepatic failure

The protective effects of the deleted form of HGF against the reduction in fibrinogen, indicating its consumption, the prolongation of hepaplastin-test time, indicating coagulopathy and the elevation of γ-glutamyl transpeptidase, indicating cholangitis, induced by *P. acnes* + *S. ty-*

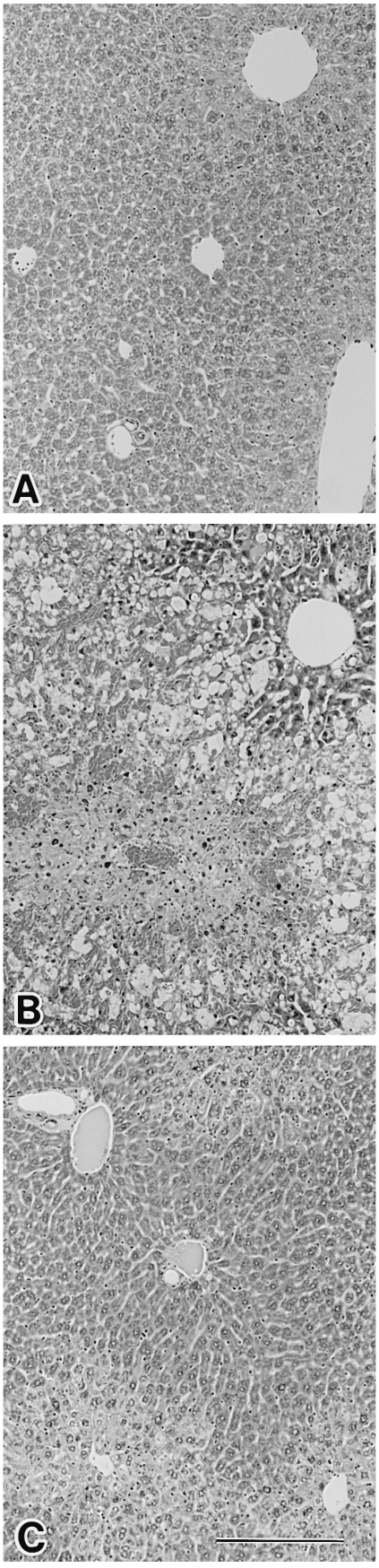


Fig. 1. Histological appearance of the liver from a mouse after a single i.v. injection of dimethylnitrosamine. Normal (A), control (B) and dHGF-treated (C). Paraffin-embedded sections were stained with hematoxylin and eosin (×30).

phosa endotoxin are summarized in Table 1. In control rats, the fibrinogen level was reduced from 1.98 to 0.38 g/l, the hepaplastin-test time was prolonged from 30.9 to 48.7 s and serum γ-glutamyl transpeptidase level was elevated from 2.3 to 7.0 U/l. In rats treated with the deleted form of HGF (0.5 mg/kg i.v. twice daily for 7 days before the administration of *S. typhosa* endotoxin), hepaplastin-test time, fibrinogen level and serum γ-glutamyl transpeptidase level were reversed to 0.55 g/l, 35.6 s and 5.2 U/l, respectively (Table 1). The deleted form of HGF prevented the prolongation of hepaplastin-test time, the reduction in fibrinogen level and the elevation of γ-glutamyl transpeptidase level induced by *S. typhosa* endotoxin in rats.

The effect of the deleted form of HGF on the mortality of mice with severe hepatic failure induced by endotoxin was investigated. Of the control mice injected with *P. acnes* + *S. typhosa* endotoxin, galactosamine + *E. coli* en-

Table 3
Effects of dHGF on prolongation of hepaplastin test time, increase in serum level of aspartate aminotransferase (AST) and decreases in hepatic glutathione (GSH) content and hepatic superoxide dismutase activity (SOD) of mice receiving a lethal dose of dimethylnitrosamine or acetaminophen

			Hepaplastin (s)	AST (U/l)	GSH (μmol/g liver)	SOD (mg/g liver)
Normal			17.0 ± 0.0	42 ± 2	9.7 ± 0.3	1.80 ± 0.05
Dimethylnitrosamine	vehicle		21.9 ± 2.2	810 ± 252	4.1 ± 0.6	0.89 ± 0.02
	dHGF	0.3 mg/kg	17.7 ± 0.4[a]	163 ± 42[b]	6.7 ± 0.2[b]	1.33 ± 0.03[b]
		1.5 mg/kg	18.1 ± 0.7[a]	211 ± 131[b]	6.0 ± 0.4[b]	1.32 ± 0.07[b]
		7.5 mg/kg	16.6 ± 0.1[b]	51 ± 8[b]	6.8 ± 0.3[b]	1.57 ± 0.05[b]
Acetaminophen	vehicle		19.5 ± 1.0	704 ± 333	0.5 ± 1.0	0.72 ± 0.20
	dHGF	0.3 mg/kg	17.4 ± 0.1[b]	344 ± 108	1.5 ± 0.5	1.13 ± 0.08
		1.5 mg/kg	17.1 ± 0.2[b]	177 ± 67[a]	0.5 ± 0.1	1.35 ± 0.05[a]
		7.5 mg/kg	16.5 ± 0.3[b]	176 ± 109[a]	1.2 ± 0.2	1.47 ± 0.22[a]

Each value represents the mean ± S.E.M. for 5 mice.
[a] $P < 0.05$, [b] $P < 0.01$ significant differences from control group receiving vehicle.

Table 4
Effects of dHGF on elevated levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP), bilirubin and free cholesterol and on a reduction of bromosulphalein (BSP) clearance after the administration of α-naphthylisothiocyanate in rats

	Normal	α-Naphthylisothiocyanate cholangiolitic hepatopathy		
		vehicle	dHGF (mg/kg)	
			0.15	0.5
AST (U/l)	84 ± 4	236 ± 13	153 ± 22[a]	110 ± 10[a]
ALT (U/l)	15 ± 1	75 ± 8	45 ± 8[a]	35 ± 3[a]
ALP (U/l)	633 ± 24	1326 ± 69	995 ± 50[a]	980 ± 59[a]
γ-GTP (U/l)	4.2 ± 0.1	8.7 ± 0.5	8.1 ± 0.5	6.3 ± 0.2[a]
Bilirubin (mg/dl)	0.65 ± 0.02	2.95 ± 0.26	2.63 ± 0.33	1.53 ± 0.09[a]
F-cholesterol (mg/dl)	19 ± 1	73 ± 5	71 ± 5	52 ± 2[a]
BSP clearance test (%)	0.5 ± 0.0	8.2 ± 1.0	7.6 ± 1.5	2.5 ± 0.3[a]

Each value represents the mean ± S.E.M. for 10 rats.
[a] $P < 0.01$ significantly different from control group receiving vehicle.

Table 5
Protective effects of dHGF against the hepatic fibrosis induced by repeated administration of α-naphthylisothiocyanate in mice

	Normal ($n = 20$)	α-Naphthylisothiocyanate fibrosis	
		vehicle ($n = 14$)	dHGF 2.5 mg/kg ($n = 14$)
Aspartate aminotransferase (U/1)	38 ± 1	73 ± 7	31 ± 1[b]
Alanine aminotransferase (U/1)	13 ± 0	41 ± 4	12 ± 1[b]
Free-cholesterol (mg/d1)	12 ± 0	38 ± 3	27 ± 1[b]
Glucose (mg/dl)	194 ± 7	144 ± 5	181 ± 8[b]
Hydroxyproline (μg/g liver)	99 ± 6	276 ± 14	162 ± 9[b]
Body weight (g)	23.1 ± 0.2	21.4 ± 0.3	22.3 ± 0.3[a]

Each value represents the mean ± S.E.M.
[a] $P < 0.05$, [b] $P < 0.01$ significant difference from control group receiving vehicle.

dotoxin or a lethal dose of *E. coli* endotoxin, only 0, 5 or 3 out of 25 animals survived, respectively. After pretreatment with the deleted form of HGF (3 mg/kg i.v. twice daily for 4 days) before the injections of *S. typhosa* endotoxin, galactosamine + *E. coli* endotoxin or a lethal dose of *E. coli* endotoxin, 10, 19 or 14 out of 25 animals survived, respectively (Table 2).

3.2. Hepatotoxin-induced hepatic failure

The administration of dimethylnitrosamine to mice prolonged hepaplastin-test time, elevated serum aspartate aminotransferase level (as an index of hepatocellular necrosis) and reduced GSH level and superoxide dismutase activity (act as a detoxifying system) in liver. Treatment of the animals with the deleted form of HGF (0.3–7.5 mg/kg i.v. twice daily) prevented the morphological liver injury, the prolongation of plasma clotting time, the elevation of aspartate aminotransferase, the reduction in hepatic GSH level and/or hepatic superoxide dismutase activity (Fig. 1, Table 3). The administration of acetaminophen to mice prolonged the hepaplastin-test time, elevated serum aspartate aminotransferase level and reduced the hepatic superoxide dismutase activity similar to that seen in the dimethylnitrosamine-induced model. The reduction in hepatic GSH level was more severe in the acetaminophen-induced model than in the dimethylnitrosamine-induced model. The treatment with the deleted form of HGF (0.3–7.5 mg/kg i.v. twice daily) reversed the hepaplastin-test time, serum aspartate aminotransferase level and hepatic superoxide dismutase activity in mice receiving a lethal dose of acetaminophen, while it scarcely changed the hepatic GSH content (Table 3). The effect of the deleted form of HGF on mortality of mice with severe hepatic failure induced by both agents was investigated. Of the control mice receiving a lethal dose of dimethylnitrosamine or acetaminophen, only 0 or 8 out of 25 animals survived, respectively. After pretreatment with the deleted form of HGF (3 mg/kg i.v. twice daily for 4 days), all animals survived (Table 2).

The administration of α-naphthylisothiocyanate to rats induced a moderate acute periportal cholangiolitic hepatopathy which caused clinico-chemical abnormalities such as elevated levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase and total bilirubin and pronounced hepatic-functional abnormalities, such as a reduction of bromosulphalein clearance and hypercholesteremia. The treatment with the deleted form of HGF (0.15–0.5 mg/kg i.v. twice a day) dose dependently reversed the levels of aspartate aminotransferase, alanine aminotransferase, alkaline phos-

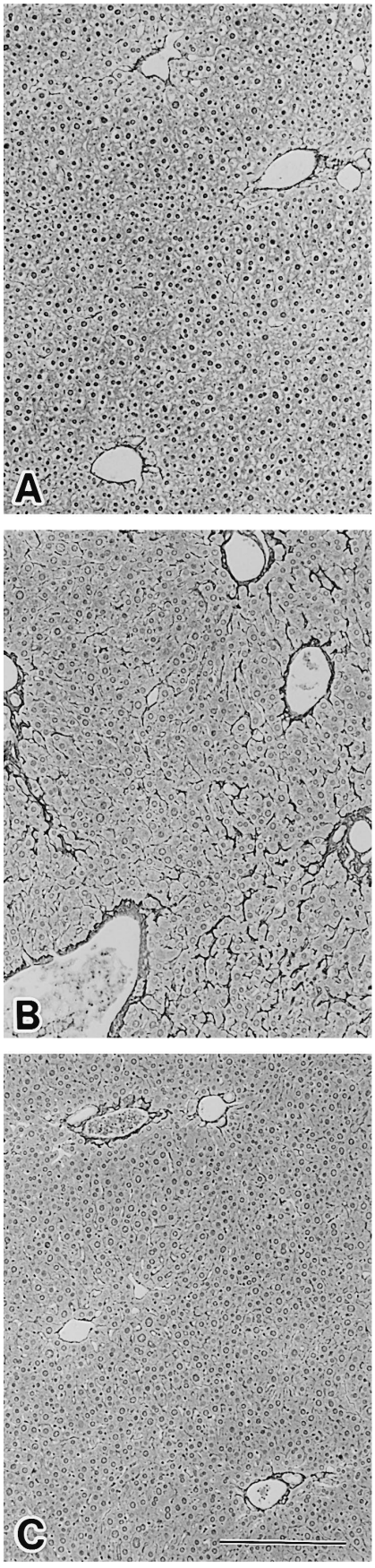


Fig. 2. Histological appearance of fibrosis in the liver from a mouse after the repeated p.o administration of a small dose of α-naphthylisothiocyanate. Normal (A), control (B) and dHGF-treated (C). Paraffin-embedded sections were stained by reticulin silver impregnation (×30).

phatase, γ-glutamyl transpeptidase and total bilirubin (as indices of liver injury) and ameliorated the reduction in bromosulphalein clearance and hypercholesteremia (as indices of functional abnormalities caused by α-naphthylisothiocyanate-induced cholangiolitic hepatopathy) (Table 4).

3.3. Chronic liver damage

Repeated administration of α-naphthylisothiocyanate to mice elevated the serum levels of alanine aminotransferase, aspartate aminotransferase and free-cholesterol and reduced the serum glucose level and body weight gain. Hepatic fibrosis and accumulation of hepatic collagen (expressed as hydroxyproline content) were observed in the control mice receiving α-naphthylisothiocyanate. The mice given the deleted form of HGF (2.5 mg/kg i.v. 5 times a week for 12 weeks) gained more weight than the control group (Table 5). Fibrosis was scarcely seen in the liver lobular and its structure closely resembled that of a normal liver (Fig. 2). The hepatic hydroxyproline content in the deleted form of HGF-treated group was lower than that in the control group. The deleted form of HGF also prevented the increases in levels of alanine aminotransferase, aspartate aminotransferase and free-cholesterol and the reduction of serum glucose.

Repeated administration of a small dose of dimethylnitrosamine to rats induced liver cirrhosis and severe hepatic disorders. Histological examination revealed that fibrosis existed principally in the centrilobular zone, but extended into the parenchyma along the sinusoids in the liver of the control rats (Fig. 3). In animals with severe cirrhosis, fibrous septum formation was often seen between central veins. Inflammatory cell infiltration, congestion, hemorrhagic necrosis and brown pigmentation derived from stale hemorrhage, which were seen centrally in fibrous stroma, varied in degree. In these animals, prolongation of the plasma clotting time, increases in the levels of alanine aminotransferase, aspartate aminotransferase, total bilirubin and free-cholesterol and a reduction in platelets, glucose, hepatic content of glycogen and GSH were observed on day 28 (Table 6). In the animals treated with the deleted form of HGF (0.05–0.5 mg/kg i.v. twice a day), slight fibrosis was seen only in a limited area around central veins and hepatocellular swelling, hemorrhagic necrosis, congestion brown pigmentation and inflammatory cell infiltration were hardly noticeable (Fig. 3). The deleted form of HGF dose dependently prevented the prolongation of plasma clotting time, thrombocytopenia, increase in the levels of aspartate aminotransferase, alanine aminotrans-

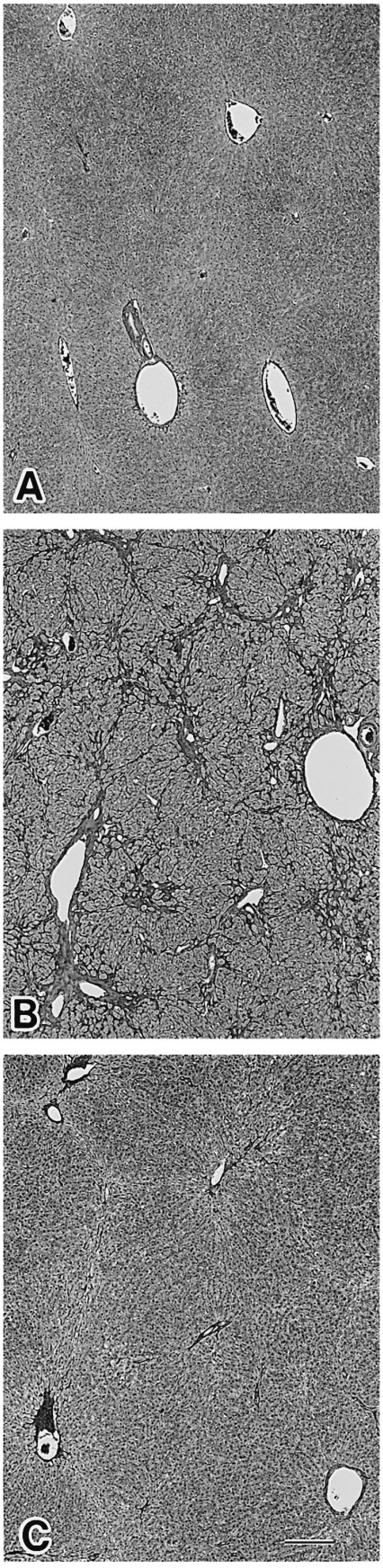


Fig. 3. Histological appearance of fibrosis in the liver from a rat after the repeated i.p. injection of a small dose of dimethylnitrosamine. Normal (A), control (B) and dHGF-treated (C). Paraffin-embedded sections were stained by reticulin silver impregnation (×12).

ferase and total bilirubin, the reduction in glucose, hepatic content of GSH and glycogen and hepatic fibrosis, which were induced by dimethylnitrosamine (Table 6). When the treatment with the deleted form of HGF (0.5 mg/kg i.v. twice a day) was started on day 28 after cirrhosis had been established with dimethylnitrosamine, it rapidly ameliorated blood coagulopathy, hypoproteinemia, abnormalities of levels of alanine aminotransferase, aspartate aminotransferase and total bilirubin (Table 7).

3.4. Coagulopathy in acute hepatic failure

The effect of the deleted form of HGF on coagulopathy in acute hepatic failure was studied by using rats with 70% partial resection of normal or cirrhotic liver. A model of acute-on-chronic hepatic failure model was achieved by performing 70% hepatectomy of the cirrhotic liver. Cirrhosis was induced by the repeated administration of carbon tetrachloride (CCl_4) (1 ml/kg p.o. twice a week for 12

Table 6
Protective effects of dHGF against coagulopathy, thrombocytopenia and clinico-chemical abnormalities in rats with liver cirrhosis induced by the repeated injection of dimethylnitrosamine

	Normal	Dimethylnitrosamine-induced cirrhosis		
		vehicle	dHGF 0.05 mg/kg	dHGF 0.5 mg/kg
Hepaplastin (s)	25.8 ± 0.2	178.6 ± 36.3	47.1 ± 13.8[b]	28.5 ± 0.4[b]
Platelets ($10^3/\mu l$)	982 ± 55	319 ± 45	586 ± 114[a]	1064 ± 52[b]
AST (U/l)	71 ± 2	193 ± 18	105 ± 9[b]	78 ± 3[b]
ALT (U/l)	18 ± 1	64 ± 5	38 ± 3[b]	26 ± 1[b]
Bilirubin (mg/dl)	0.29 ± 0.02	1.56 ± 0.29	0.55 ± 0.19[b]	0.29 ± 0.02[b]
Free-cholesterol (mg/dl)	21 ± 1	40 ± 5	22 ± 1[b]	28 ± 2[a]
Glucose (mg/dl)	180 ± 5	108 ± 6	134 ± 7[b]	158 ± 5[b]
Glycogen (mg/g liver)	41.6 ± 1.6	24.6 ± 4.6	38.0 ± 3.3[b]	41.5 ± 1.6[b]
GSH (μmol/g liver)	4.87 ± 0.16	2.54 ± 0.33	4.39 ± 0.18[b]	5.11 ± 0.14[b]
Hydroxyproline (μg/g liver)	119 ± 4	742 ± 59	433 ± 50[b]	313 ± 27[b]

Each value represents the mean ± S.E.M. for 8–10 rats.
[a] $P < 0.05$, [b] $P < 0.01$ significant difference from control group receiving vehicle.

Table 7
Effects of treatment with dHGF on coagulopathy, hypoproteinemia and clinico-chemical abnormalities in rats with liver cirrhosis induced by dimethylnitrosamine

	Normal ($n = 13$)	Dimethylnitrosamine-induced cirrhosis	
		vehicle ($n = 7$)	dHGF 0.5 mg/kg ($n = 9$)
Hepaplastin (s)	26.9 ± 0.3	192.1 ± 39.7	64.5 ± 11.4[b]
Fibrinogen (g/l)	2.37 ± 0.04	1.00 ± 0.11	1.84 ± 0.08[b]
Total protein (g/dl)	6.4 ± 0.1	3.4 ± 0.4	5.1 ± 0.2[b]
HDL-cholesterol (mg/dl)	48 ± 3	14 ± 3	32 ± 3[b]
AST (U/l)	71 ± 3	229 ± 38	125 ± 8[b]
ALT (U/l)	17 ± 1	64 ± 5	47 ± 4[b]
Bilirubin (mg/dl)	0.28 ± 0.02	2.09 ± 0.47	1.24 ± 0.16[a]
Liver weight (g/100 g BW)	4.18 ± 0.05	1.78 ± 0.22	2.65 ± 0.33[a]

Each value represents the mean ± S.E.M.
[a] $P < 0.05$, [b] $P < 0.01$ significant difference from control group receiving vehicle.

Table 8
Effects of dHGF infusion on coagulopathy and decreases in total protein, HDL-cholesterol, hepatic GSH content and liver weight until 48 h after 70% partial resection of normal- and cirrhotic-liver

	Intact ($n = 16$)	Normal liver hepatectomy			Cirrhotic liver hepatectomy		
		vehicle ($n = 32$)	dHGF (mg/kg/day)		vehicle ($n = 20$)	dHGF (mg/kg/day)	
			0.1 ($n = 15$)	0.3 ($n = 15$)		0.1 ($n = 16$)	0.3 ($n = 16$)
Hepaplastin	29.4 ± 0.4	34.7 ± 0.7	32.3 ± 1.3[a]	31.5 ± 0.8[b]	42.8 ± 4.7	34.4 ± 2.5	32.3 ± 1.4[a]
Total protein	6.0 ± 0.1	5.8 ± 0.1	6.3 ± 0.1[b]	6.4 ± 0.2[b]	4.4 ± 0.1	4.7 ± 0.1[a]	4.7 ± 0.1[a]
HDL-chol.	36.9 ± 1.5	21.2 ± 0.8	27.4 ± 1.6[b]	31.3 ± 1.6[b]	26.5 ± 2.1	34.4 ± 2.0[a]	36.5 ± 3.4[b]
GSH	2.5 ± 0.2	2.5 ± 0.2	2.7 ± 0.3	4.9 ± 0.3[b]	0.3 ± 0.1	0.5 ± 0.1	0.9 ± 0.2[a]
Liver weight	3.3 ± 0.1	2.1 ± 0.0	2.3 ± 0.0[b]	2.6 ± 0.1[b]	2.4 ± 0.1	3.0 ± 0.1[b]	3.1 ± 0.1[b]

Hepaplastin (s); total protein (g/dl); HDL-cholesterol (mg/dl); GSH (μmol/g-liver); liver weight (g/100 g BW); each value represents the mean ± S.E.M.
[a] $P < 0.05$, [b] $P < 0.01$ significant difference from control group receiving vehicle.

weeks). Partial resection of the cirrhotic liver caused more severe hypoproteinemia and coagulopathy than resection of normal liver did. When the deleted form of HGF (0.1 and 0.3 mg/kg per day) was infused into animals just after the hepatectomy for 48 h, the plasma clotting time, total protein, HDL-cholesterol, hepatic GSH level and liver weight were rapidly reversed as well as those in the hepatectomized rats with a normal liver. However, the decrease in serum total protein and hepatic GSH content still remained after the infusion of the deleted form of HGF (Table 8).

3.5. Spontaneous fulminant hepatic failure model Long–Evans cinnamon rats

Long–Evans cinnamon rats were used from just after the onset of jaundice and bilirubinuria and were a model of fulminant hepatic failure. The initial levels of serum aspartate aminotransferase and alanine aminotransferase were higher than 450 and 150 U/l in Long–Evans cinnamon rats, while normal levels of serum aspartate aminotransferase and alanine aminotransferase were around 100 and 25 U/l. The deleted form of HGF (1 mg/kg per day) or vehicle was constantly infused to Long–Evans cinnamon rats through a catheter placed in the jugular vein by using an infusion pump. The infusion was started just after the onset of jaundice. In control Long–Evans cinnamon rats receiving vehicle, 20 out of 21 animals died 4 days after the onset of jaundice (Fig. 4). In the surviving Long–Evans cinnamon rat receiving vehicle, hepaplastin test time (as an index of coagulopathy) was longer than that in the survivors receiving the deleted form of HGF. After the infusion of the deleted form of HGF (1 mg/kg per day for 4 days), 7 out of 20 Long–Evans cinnamon rats survived.

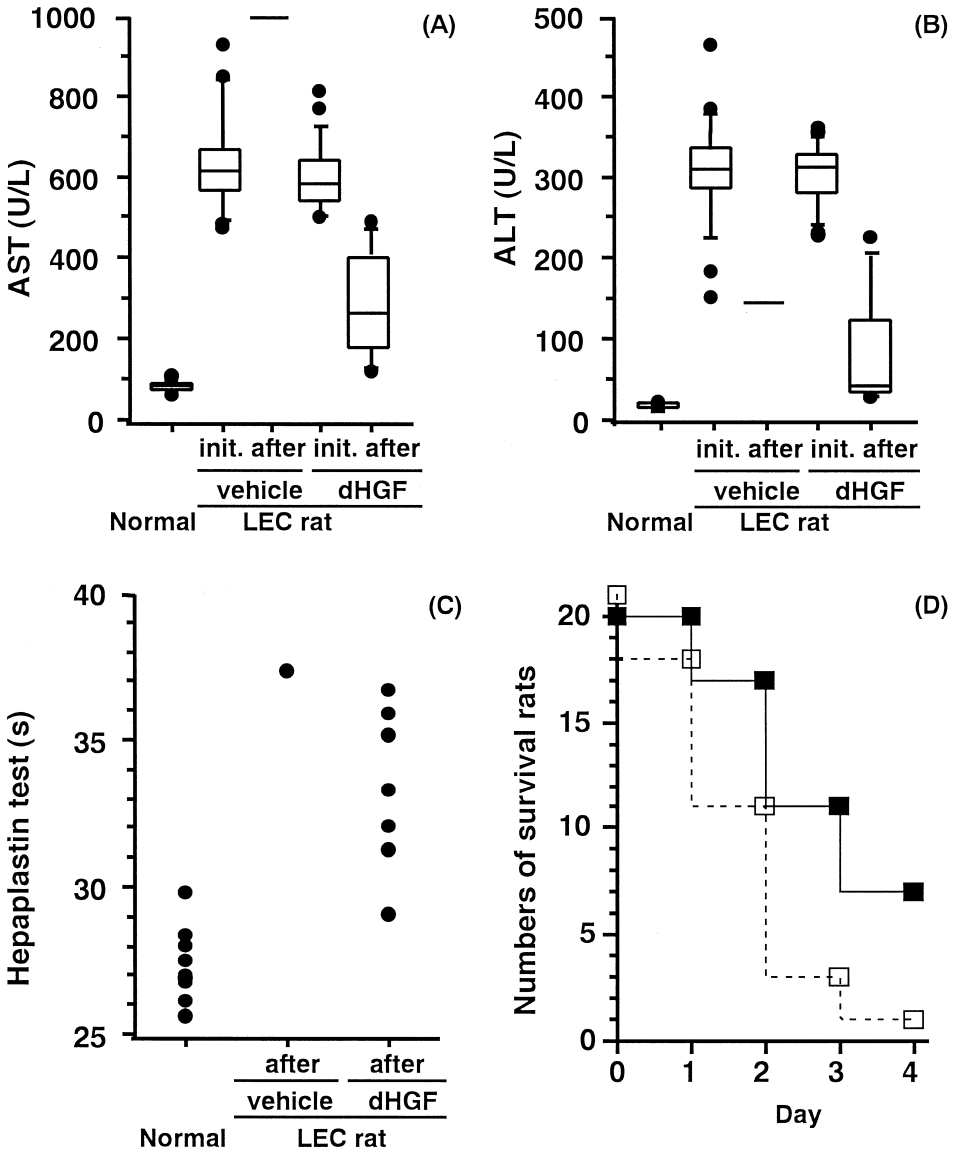
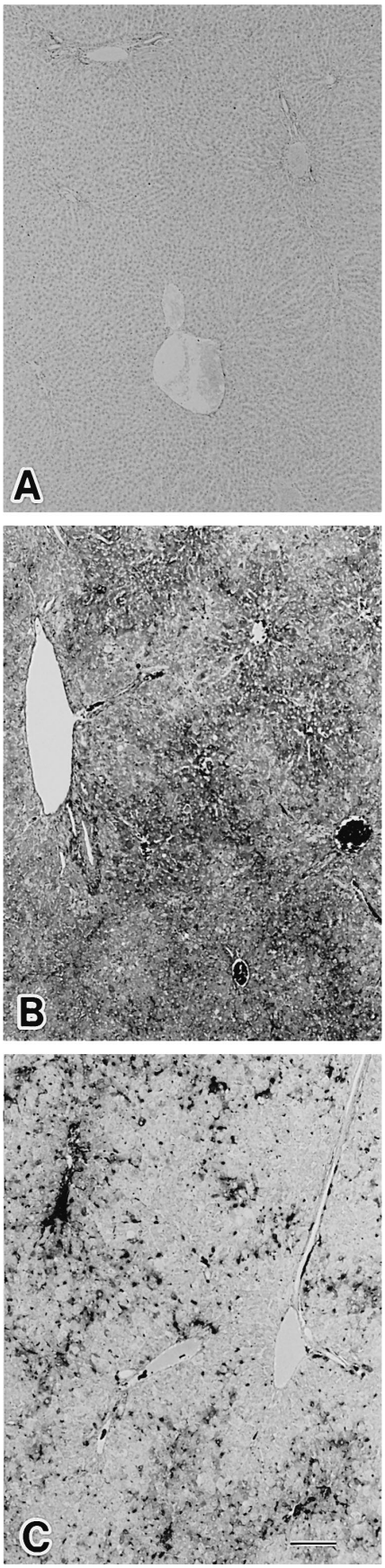


Fig. 4. Effects of dHGF infusion on serum levels of aspartate aminotransferase; AST (A) and alanine aminotransferase; ALT (B), plasma clotting time (C) and survival rate (D) on Long–Evans cinnamon (LEC) rats after the onset of jaundice. Each box-plot or scatter plot represents the value for surviving rats.



Histological examination revealed that many new hepatocytes, which had not accumulated excessive copper, were formed in the portal vein area of the Long–Evans cinnamon rats receiving the deleted form of HGF (Fig. 5).

4. Discussion

The liver plays a central role in the synthesis and clearance of most clotting proteins. Coagulopathy is due to an impaired synthesis of coagulating proteins, the production of abnormal proteins and disseminated intravascular coagulation (Rodzynek et al., 1984; Mombelli et al., 1993; Robson et al., 1993). In patients, an impaired hepatic synthesis of vitamin K-dependent clotting factors is a main risk factor for multiple, potentially lethal, complications in terminal-stage liver disease (Boks et al., 1986). Fujiwara et al. (1988) have reported that rats with acute liver failure induced by dimethylnitrosamine mainly died of a disordered blood coagulability because the survival time was negatively correlated with plasma clotting time after dimethylnitrosamine treatment. Accordingly, we studied the effect of the deleted form of HGF on the disordered plasma coagulability of rats and mice. Partial resection of cirrhotic liver (induced by CCl_4) or dimethylnitrosamine administration caused severe coagulopathy and hypoproteinemia in rats. The deleted form of HGF ameliorated the disordered plasma coagulability even in these severe conditions. Moreover, the treatment with the deleted form of HGF prevented the reduction in fibrinogen (indicating its consumption), the prolongation of hepaplastin time (indicating coagulopathy) and the elevation of γ-glutamyl transpeptidase (indicating cholangitis) in the rats induced by *P. acnes* + *S. typhosa* endotoxin. Further, the treatment with the deleted form of HGF reduced the mortality of mice with severe coagulopathy induced by the administration of *P. acnes* plus *S. typhosa* endotoxin, galactosamine plus *E. coli* endotoxin and a lethal dose of *E. coli* endotoxin, acetaminophen or dimethylnitrosamine. The deleted form of HGF treatment probably accelerates the hepatic synthesis of vitamin K-dependent clotting proteins to compensate for the consumption of coagulating factors and thus averts death caused by these hepatotoxins. Long–Evans cinnamon rats have been established as a mutant strain suffering from fulminant hepatitis and severe jaundice at about 4 months of age (Sasaki et al., 1985; Yoshida et al., 1987). In control Long–Evans cinnamon rats receiving vehicle, 20 out of 21 animals died 4 days after the onset of jaundice. In a Long–Evans cinnamon rat receiving vehicle, coagulopathy was severer than that in the survivors receiving the deleted form of HGF. There-

Fig. 5. Histological appearance of the liver from a surviving Long–Evans cinnamon (LEC) rat. Normal (A), control LEC (B) and dHGF-treated-LEC (C). Paraffin-embedded sections were stained by Timm's method for copper (×12).

fore, the amelioration of coagulopathy induced by the deleted form of HGF possibly contributes to the reduction in mortality after the onset of jaundice in Long–Evans cinnamon rats. These results indicate that the deleted form of HGF treatment may save the lives of patients with hepatocellular dysfunction because the deleted form of HGF may ameliorate coagulopathies associated with terminal-stage liver disease in which the synthesis of vitamin K-dependent clotting factors is impaired.

α-naphthylisothiocyanate-induced cholestasis is characterized histopathologically by edema in the portal area and infiltration of granulocytes into the walls of the interlobular bile ducts (Ungar et al., 1960; Goldfarb et al., 1962). Increased levels of total bilirubin, alkaline phosphatase and cholesterol, which are markers of cholestasis, have been reported in rats after administration of a single dose of α-naphthylisothiocyanate (Roberts and Plaa, 1965). Roos et al. (1992) have reported that HGF prevents the increases in levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and bilirubin which are indices of liver injury in this model, but did not demonstrate whether HGF ameliorates the hepatic functional abnormalities such as the reduction in bromosulphalein clearance and hypercholesteremia. It is noted that the deleted form of HGF not only prevented liver injury and the associated increase in levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase and total bilirubin but also ameliorated the hepatic functional abnormalities and the associated decrease in bromosulphalein clearance and hypercholesteremia in α-naphthylisothiocyanate-induced cholangiolitic hepatopathy.

The increases in levels of aspartate aminotransferase and alanine aminotransferase were consistent with the morphological findings of hepatocellular necrosis in animals receiving repeated administration of α-naphthylisothiocyanate or dimethylnitrosamine. The deleted form of HGF prevented or ameliorated the abnormalities of these parameters and morphological features in these models. It is well known that many hepatotoxins lead to hepatic fibrosis (Baraona et al., 1993). Repeated administration of α-naphthylisothiocyanate elevates serum levels of aspartate aminotransferase, alanine aminotransferase and free-cholesterol, leading to hepatic fibrosis. The deleted form of HGF reduced the liver injury induced by α-naphthylisothiocyanate because it prevented the increases in levels of alanine aminotransferase, aspartate aminotransferase and free-cholesterol and the decrease in serum glucose. In the mice receiving the deleted form of HGF, hepatic fibrosis was scarcely observed and the body weight was greater than that of control mice. The mechanism by which the deleted form of HGF exerts this preventive effect remains to be elucidated, but the preventive effects of the deleted form of HGF should result in reduced hepatic fibrosis. Dimethylnitrosamine is activated by microsomal enzymes (Mostafa et al., 1981). It appears to block liver protein synthesis and to act primarily as a liver poison, producing severe liver necrosis in animals. Repeated injection of dimethylnitrosamine to rats markedly increased the serum enzyme activities related to liver function and caused severe thrombocytopenia, hypoproteinemia and coagulopathy. This thrombocytopenia has been explained by consumption of platelets due to intravascular coagulation associated with fibrin formation in the sinusoids (Hirata et al., 1989). Treatment with the deleted form of HGF drastically prevented the abnormalities of the clinico-chemical parameters and reversed the reduction in serum glucose and hepatic glycogen content. In the animals treated with the deleted form of HGF, slight fibrosis was seen only in a limited area around central veins, and hepatocellular swelling, hemorrhagic necrosis, congestion brown pigmentation and inflammatory cell infiltration were hardly remarkable. One possible explanation for this beneficial effect is that the normalization of hepatic protein synthesis induced by the deleted form of HGF stimulates the restoration of hepatic function. When given to rats simultaneously with dimethylnitrosamine, the deleted form of HGF prevented the accumulation of excessive collagen and inflammatory cells. Inflammatory and immune cells secrete factors that activate collagen biosynthesis and fibroproliferative inflammation is currently considered to be a prerequisite for fibrosis (Matsuoka et al., 1989). Thus the data suggest that the deleted form of HGF prevents liver fibrosis by reducing the inflammatory reaction. Such a benefit may be generated by protection against liver injury and by stimulation of recovery from injury by promotion of the proliferation of the remaining hepatocytes or restoration of function of injured hepatocytes.

It is interesting to note that the deleted form of HGF is capable of inhibiting such a diverse array of hepatic injuries. The protective mechanism may be explained partially by increasing hepatic detoxication because the deleted form of HGF accelerates hepatocyte proliferation and increases liver size (Shima et al., 1994; Masunaga et al., 1996). The hepatic GSH content is an important marker of hepatocellular condition and hepatic detoxication capacity. The consumption of GSH reflects hepatocellular dysfunctions such as coagulopathy, clinico–chemical abnormalities or morphological liver injury (Frei et al., 1985; Strolin Benedetti et al., 1975; Liu et al., 1993). For example, the reduction in hepatic GSH content in mice receiving dimethylnitrosamine or acetaminophen is suggestive of the massive hepatic necrosis. Furthermore, the GSH content of a dimethylnitrosamine-induced cirrhotic liver or of a remnant of cirrhotic liver after partial hepatectomy was lower than that of an intact liver or of a remnant normal liver. Treatment with the deleted form of HGF prevented the reduction in hepatic GSH content in mice receiving a lethal dose of dimethylnitrosamine. Frei et al. (1985) have reported that hepatic GSH can act as a potential detoxifying system for the alkylating species of dialkylnitrosoamines. When the activities of hepatic GSH and

glutathione *S*-transferase were enhanced, the degree of methylation of guanine by dimethylnitrosamine was greatly reduced, because GSH inhibits the alkylation of liver DNA by dimethylnitrosamine in vivo. However, the hepatic GSH content of mice receiving acetaminophen can be completely depleted as a result of metabolism of acetaminophen (Frei et al., 1985). In rats with liver cirrhosis induced by dimethylnitrosamine and in rats after the partial resection of normal and cirrhotic liver, the hepatic GSH content was dose dependently elevated by the deleted form of HGF. Restoration of hepatic GSH content by the treatment with the deleted form of HGF might provide protection against hepatotoxicity caused by various agents. The deleted form of HGF also dose dependently prevented the reduction in hepatic superoxide dismutase activity in the mice receiving dimethylnitrosamine and acetaminophen. Particularly, in the mice receiving a lethal dose of acetaminophen, hepatic superoxide dismutase might play an important role because the deleted form of HGF reduced mortality in spite of the depletion of hepatic GSH. Arthur et al. (1985) described that extracellular release of superoxide from macrophages is the most important source of oxygen-derived free radicals in severe hepatic injury and that hepatic superoxide dismutase, which metabolizes superoxide in the extracellular space, reduces the degree of hepatic necrosis. The mechanisms by which the deleted form of HGF elevates hepatic GSH and superoxide dismutase remain to be elucidated, but this is the first demonstration of the effect of the deleted form of HGF in preventing the reduction in superoxide dismutase activity and GSH content in injured liver. Finally, it remains to be seen whether HGF has the same protective effect because HGF could not be supplied in sufficient amount to enable it to be compared with the deleted form of HGF in this study. It is concluded that the deleted form of HGF prevents various liver injuries, reduces mortality caused by hepatic disorders, ameliorates morphological damage and reverses hepatic dysfunction in various models of liver injury that give rise to acute or chronic hepatotoxic events. These results indicate that the deleted form of HGF could have a therapeutic effect in patients with severe hepatic failure.

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