

# Repeated intravenous injection of recombinant human hepatocyte growth factor ameliorates liver cirrhosis but causes albuminuria in rats

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**Abstract.** Hepatocyte growth factor (HGF) is a promising agent for the treatment of liver cirrhosis because of its mitogenic and anti-fibrotic effects. We investigated the effect of recombinant human HGF (rh-HGF) on cirrhosis development; its pharmacokinetics and nephrotoxicity in rats with liver cirrhosis induced by 4-week treatment with dimethylnitrosamine (DMN). rh-HGF (0.3 mg/kg) was intravenously administered to rats once a day for 4 weeks in parallel with DMN treatment or twice a day for the last 2 weeks of DMN treatment. Repeated doses of rh-HGF increased the liver weight and serum albumin, and reduced serum ALT. The development of hepatic fibrosis was inhibited more efficiently by extended low-dose treatment with rh-HGF. In cirrhotic rats, serum levels of rh-HGF increased and clearance was decreased, leading to an increase in the area under the plasma-concentration time curve and a decrease in the steady-state volume of distribution. Repeated doses of rh-HGF led to increased urinary albumin excretion, but no rh-HGF-treated animals developed increased serum creatinine levels. Urinary albumin excretion returned to baseline after the cessation of rh-HGF. These results suggest that extended treatment with rh-HGF is required for the attenuation of cirrhosis, and repeated doses of rh-HGF cause adverse effects in extra-hepatic organs. These issues must be resolved before the widespread application of rh-HGF in the treatment of liver cirrhosis.

## Introduction

Hepatocyte growth factor (HGF) was originally identified as a potent hepatocyte mitogen from the plasma of patients with fulminant hepatic failure (1,2). We have established an enzyme-linked immunosorbent assay (ELISA) to measure human HGF in the serum, and we identified increased serum HGF levels in patients with various liver diseases (3). Additionally, serum HGF levels are a valuable prognostic tool in fulminant hepatic failure (4).

HGF is a multifunctional growth factor that acts as a mitogen, motogen, and morphogen for a variety of cells, including epithelial and endothelial cells (5-8). It is a major agent promoting hepatocyte proliferation (9-11) and acts in concert with transforming growth factor (TGF)- $\alpha$  and heparin-binding epidermal growth factor during liver regeneration (12,13). Additionally, HGF ameliorates hepatic injury via anti-apoptotic effects in animal models of fulminant hepatic failure (14-20) and attenuates hepatic fibrosis in animals with liver cirrhosis (21-25). Consequently, HGF is considered to not only induce liver regeneration but also to inhibit disease progression and ameliorate hepatic fibrosis in patients suffering from intractable liver diseases. Therefore, the clinical uses of recombinant human HGF (rh-HGF) in the treatment of fatal liver diseases, including fulminant hepatic failure, small-for-size grafts in living donor liver transplantation, and liver cirrhosis, are currently under investigation in Japan.

We recently showed that bolus intravenous injection of rh-HGF led to increased serum levels of human HGF, and intravenously administered HGF was primarily distributed to the liver (26). Additionally, despite its short half-life, a single intravenous injection of rh-HGF induced tyrosine phosphorylation of c-Met, a specific receptor for HGF, in liver tissue. However, intravenously administered rh-HGF is thought to be primarily metabolized in the liver, but the effect of liver injury on the pharmacokinetics of rh-HGF is not well understood.

In the present study, we investigated the effect of intravenously administered rh-HGF on hepatic fibrosis and its

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pharmacokinetics in a rat model of liver cirrhosis. We further examined changes in urinary albumin excretion, a known adverse effect in extra-hepatic organs caused by the repeated administration of rh-HGF.

### Materials and methods

**Animals.** Seven-week-old male SD rats were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were maintained under a constant room temperature of 25°C and given free access to water and the indicated diet throughout the study. The protocol for animal studies was approved by the Ethics Committee of University of Miyazaki, Faculty of Medicine (Miyazaki, Japan). All animal experiments were performed after a 1-week acclimation period on a standard diet.

To induce liver cirrhosis, 1% dimethylnitrosamine (DMN) (Wako Pure Chemical Industries Ltd., Tokyo, Japan) dissolved in saline was given intraperitoneally at 1 ml per kg body weight for three consecutive days per week for 4 weeks. Rats were divided into four groups as follows: group C1 (n=7), rats treated with intravenous injection of phosphate-buffered saline (PBS) once a day for 4 weeks in parallel with DMN treatment; group H1 (n=8), rats treated with intravenous injection of rh-HGF (0.3 mg/kg) once a day for 4 weeks in parallel with DMN treatment; group C2 (n=9), rats treated with intravenous injection of PBS twice a day for the latter 2 weeks of DMN treatment; group H2 (n=10), rats treated with intravenous injection of rh-HGF (0.3 mg/kg) twice a day for the latter 2 weeks of DMN treatment.

After 4-week treatment with DMN, rats were sacrificed and serum albumin and alanine aminotransferase (ALT) were examined. The liver, spleen, and kidneys were immediately excised, and the wet weight of these organs was determined. Samples were subjected to histological analysis or frozen in liquid nitrogen and stored at -80°C until analysis. TGF- $\beta$  levels in liver tissue were measured by a commercially available ELISA kit (R&D Systems Co., MN, USA).

Seventy-percent partial hepatectomy was performed according to a modification of the method of Higgins and Anderson (27). The rats were anesthetized with ether and a two-thirds partial hepatectomy was performed.

**Measurement of serum human HGF.** A silicone-rubber catheter (0.5x1.0 mm o.d.) was inserted into the jugular vein of normal, partially hepatectomized, or DMN-induced cirrhotic rats, and saline was administered continuously via the catheter using an infusion pump (0.1 ml/h) to prevent obstruction. rh-HGF (0.1 mg/kg) was injected into the femoral vein in <10 sec, and sequential blood samples were obtained via the catheter 5, 10, 20, 30, 60, 90 and 120 min after the injection.

Serum levels of human HGF were determined using a commercially available ELISA kit (Otsuka Pharmaceutical Co., Tokushima, Japan), in which only human HGF and not rat HGF is detected (3,28). Pharmacokinetic parameters were calculated using WinNonlin (Pharsight Co., CA, USA).

**Histological examination for liver cirrhosis.** Two 5-mm thick slices from the two major liver lobes (left lateral and median lateral lobes) were fixed in 10% formalin. After routine

processing of paraffin sections, 3- $\mu$ m thick liver sections were made. To evaluate the degree of fibrosis, sections were stained with 0.1% (w/v) Sirius red F3BA (Sigma, St. Louis, MO, USA) for 1 h in a saturated aqueous solution (1.2% w/v) of picric acid (Wako); the final pH of the solution was 2.0. After staining, slides were rinsed for 2 min in 0.01 N HCl solution to remove unbound dye; following dehydration in alcohol, slides were mounted for observation. The Sirius red-positive areas were measured using an Olympus video micrometer, VM-30 (Olympus, Tokyo, Japan). The results were expressed as a fibrotic rate (%), which was calculated as the ratio of the Sirius red-positive area to the total area examined.

**Measurement of urinary albumin excretion.** Rats with DMN-induced liver cirrhosis were treated with PBS or rh-HGF as described above. Urine was periodically collected during PBS or DMN treatment, and urine albumin and creatinine were determined.

Repeated doses of rh-HGF (1.0 mg/kg/day) were intravenously administered to normal rats for 5 consecutive days per week for 2 weeks (days 1-5 and 8-12). Urinary levels of albumin and creatinine were examined during HGF treatment and for the following 2 weeks.

**Statistical analysis.** Unless otherwise specified, data are expressed as mean  $\pm$  SD. Statistical parameters were ascertained using StatView J-4.5 software (Abacus Concepts Ind., Berkeley, CA). Differences between means were assessed by using the unpaired Student's t-test. The significance level was set at  $p < 0.05$ .

### Results

**Repeated intravenous injection of rh-HGF stimulated liver regeneration and attenuated liver injury in DMN-induced cirrhotic rats.** To examine the effects of repeated intravenous injection of rh-HGF on liver regeneration, we measured the liver weights and serum albumin levels in rats with DMN-induced cirrhosis treated with either saline or rh-HGF (Table I). Treatment with rh-HGF for both 2 and 4 weeks concurrent with DMN administration significantly increased both liver weights and serum albumin levels compared with PBS-treated animals. Additionally, compared to untreated rats, serum ALT levels were elevated in DMN-induced cirrhotic rats treated with PBS (groups C1 and C2) (Table I), and treatment with rh-HGF for 2 weeks significantly decreased ALT levels (C2 vs. H2). While ALT values for animals treated with rh-HGF for 4 weeks (group H1) were reduced compared to group C1, this difference was not significant. The reason for the differences in 2- and 4-week rh-HGF treatment is unclear. Nevertheless, repeated intravenous injection of rh-HGF not only stimulated liver regeneration but also attenuated liver injury in DMN-induced cirrhotic rats.

**Prolonged treatment with rh-HGF efficiently attenuated hepatic fibrosis in DMN-induced cirrhotic rats.** Liver weight and serum ALT are good markers for liver regeneration and injury, but the pathological hallmark of cirrhosis is extensive liver fibrosis. Thus, we wished to examine the effect of rh-HGF on hepatic fibrosis. When rh-HGF was intravenously



	Group			Group		
	C1 (n=7)	H1 (n=8)	p-value	C2 (n=9)	H2 (n=10)	p-value
Body weight (BW g)	270.1±64.6	319.0±32.5	0.105	305.3±38.2	311.0±22.9	0.705
Liver (g/100 g BW)	2.77±1.10	3.91±0.40	0.034	3.32±0.93	4.65±0.52	0.003
Kidney, right (g/100 g BW)	0.42±0.09	0.39±0.05	0.405	0.41±0.07	0.43±0.06	0.584
Albumin (g/dl)	1.81±0.50	2.34±0.19	0.031	2.06±0.43	2.92±0.18	0.0002
ALT (IU/l)	136.7±99.4	78.4±18.6	0.175	94.8±34.2	67.6±12.5	0.048

Group C1, rats treated with PBS once a day for 4 weeks in parallel with DMN administration; group H1, rats treated with 0.3 mg/kg of rh-HGF once a day for 4 weeks in parallel with DMN administration; group C2, rats administered PBS twice a day for the last 2 weeks of DMN treatment; group H2, rats administered 0.3 mg/kg of rh-HGF twice a day for the last 2 weeks of DMN treatment. Results are represented as the mean ± SD.

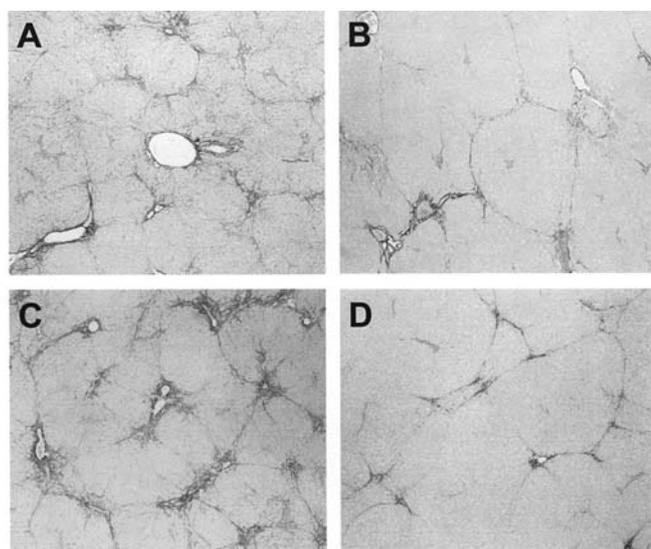


Figure 1. Representative microscopic appearance of liver tissue in DMN-induced cirrhotic rats treated with or without rh-HGF. Rats with DMN-induced cirrhosis were treated with rh-HGF for 4 or 2 weeks, and liver tissue was stained with Sirius red as described in Materials and methods. (A) Group C1; (B) Group H1; (C) Group C2; (D) Group H2 (magnification  $\times 40$ ).

injected for 4 or 2 weeks in conjunction with DMN administration, the development of hepatic fibrosis was substantially inhibited (Fig. 1). Sirius red, a dye specific for fibrotic areas, was used to quantitate the extent of liver fibrosis in PBS- or rh-HGF-treated rats (Table II). Repeated injection of rh-HGF for 4 weeks concurrent with DMN treatment (group H1) significantly reduced the areas of hepatic fibrosis compared with PBS-treated rats (group C1), but there were no significant differences between the fibrotic areas of rats treated with PBS and those of rats treated with rh-HGF for 2 weeks (groups C2 and H2, respectively). We also examined the hepatic tissue levels of TGF- $\beta$ , a key mediator of cirrhosis development (Table II). Administration of rh-HGF reduced TGF- $\beta$  levels in liver tissue, regardless of the treatment period, but these differences did not achieve statistical significance.

*Pharmacokinetics of rh-HGF administered intravenously in a bolus in normal, partially hepatectomized, and cirrhotic rats.* HGF is primarily metabolized by the liver, but patients most likely to receive rh-HGF have severe liver disease with possible impaired metabolism of a variety of compounds, including rh-HGF. Therefore, we examined changes in serum human HGF concentration sequentially after the single intravenous injection of rh-HGF in rats with normal or abnormal liver function, and we calculated pharmacokinetic parameters (Table III). When compared with normal rats, the serum levels of human HGF were elevated 5 min after injection and the terminal elimination half-life ( $T_{1/2\text{terminal}}$ ) of rh-HGF was prolonged in rats with DMN-induced cirrhosis. Consequently, an increase in the area under the plasma-concentration time curve ( $AUC_{0-\infty}$ ) and a decrease in clearance (CL) were observed in cirrhotic rats. Also, the steady-state volume of distribution ( $V_{dss}$ ) was reduced to approximately 50%, and the mean resident time ( $MRT_{0-\infty}$ ) was prolonged. The degree of pharmacokinetic change in rats with DMN-induced cirrhosis was virtually the same as that seen in rats with 70% partial hepatectomy.

*Repeated administration of rh-HGF increases albuminuria.* While most of the physiological effects of rh-HGF are confined to the liver, some changes in kidney function are seen in animals treated with rh-HGF. As a measure for kidney dysfunction, we examined urinary albumin excretion during the treatment with rh-HGF (Fig. 2). When rats were treated with rh-HGF (0.3 mg/kg, once a day) in parallel with DMN administration, the urinary albumin excretion gradually increased for 7 days from the beginning of HGF treatment, and moderate to high levels of albuminuria were observed during the following 14 days (Fig. 2A). Repeated injection of rh-HGF (0.3 mg/kg, twice a day) for days 15-28 of DMN administration rapidly increased the urinary excretion of albumin, and marked albuminuria continued throughout the experimental period. Conversely, kidney weight was not affected by either 2- or 4-week treatment with rh-HGF (Table I).

Table II. Effect of rh-HGF administration on hepatic fibrosis and hepatic levels of TGF- $\beta$  in DMN-induced cirrhotic rats.

	Group			Group		
	C1 (n=7)	H1 (n=8)	p-value	C2 (n=9)	H2 (n=10)	p-value
Fibrosis (%)	10.30 $\pm$ 5.02	4.53 $\pm$ 1.64	0.022	7.31 $\pm$ 3.84	5.71 $\pm$ 2.77	0.319
TGF- $\beta$ (ng/g tissue)	30.77 $\pm$ 10.26	22.65 $\pm$ 6.00	0.098	34.69 $\pm$ 12.06	28.03 $\pm$ 11.91	0.244

Results are represented as the mean  $\pm$  SD.

Table III. Pharmacokinetics of rh-HGF in normal, partially hepatectomized, and cirrhotic rats.

	Normal (n=4)	DMN-LC (n=5)	70% PH (n=4)
C <sub>5</sub> <sup>a</sup> (ng/ml)	89.71 $\pm$ 20.57	255.4 $\pm$ 181.4	341.2 $\pm$ 55.1
T <sub>1/2terminal</sub> (min)	27.88 $\pm$ 2.00	35.41 $\pm$ 18.74	39.49 $\pm$ 13.79
AUC <sub>0-<math>\infty</math></sub> ( $\mu$ g $\cdot$ min/ml)	1.685 $\pm$ 0.342	13.78 $\pm$ 16.48	10.84 $\pm$ 2.24
CL (ml/min/kg)	62.09 $\pm$ 13.82	20.76 $\pm$ 16.73	9.511 $\pm$ 1.864
Vdss (ml/kg)	903.7 $\pm$ 253.5	453.4 $\pm$ 174.6	419.6 $\pm$ 144.6
MRT <sub>0-<math>\infty</math></sub> (min)	14.44 $\pm$ 1.07	41.71 $\pm$ 33.20	43.79 $\pm$ 9.87

Results are represented as the mean  $\pm$  SD. <sup>a</sup>Concentration of serum human HGF 5 min after injection. DMN-LC, rats with DMN-induced liver cirrhosis; 70% PH, rats with 70% partial hepatectomy; T<sub>1/2terminal</sub>, terminal elimination half-life; AUC<sub>0- $\infty$</sub> , the area under the plasma-concentration time curve; Vdss, steady-state volume of distribution; MRT<sub>0- $\infty$</sub> , mean resident time.

To clarify whether HGF-induced albuminuria was reversible, we injected rh-HGF (1.0 mg/kg/day) via tail veins into normal rats for different periods of time, and monitored the urinary albumin excretion (Fig. 2B). Administration of rh-HGF for 5 days gradually increased albuminuria and, when rh-HGF injection was stopped on days 6 and 7, the albumin excretion reduced. When rh-HGF was subsequently administered on days 8-12, albuminuria once again worsened but, after complete cessation of rh-HGF injection, the albumin excretion gradually returned to baseline levels by days 21-28. On day 28, serum creatinine levels were unchanged compared to baseline indicating minimal permanent kidney damage (data not shown).

## Discussion

HGF is produced by mesenchymal cells as an inactive precursor, pro-HGF (29-31), and the mature protein plays important roles in the regeneration and repair of various organs, including the liver, gastrointestinal tract, and kidney. Following tissue injury, pro-HGF is converted to an active heterodimer consisting of light and heavy chains by the specific serine protease HGF activator (HGFA) (32-35). Additionally, HGFA inhibitor (HAI)-1, which was first purified as an inhibitor of HGFA (36), regulates HGFA activity at sites of tissue injury (37). Recently, Yanagida *et al* reported that the administration of recombinant human HGFA stimulated the regeneration of cirrhotic liver after partial hepatectomy through the activation of endogenous

pro-HGF (38). In the present study, we administered rh-HGF (0.3 mg/kg) in an already active heterodimeric form into cirrhotic rats once a day for 4 weeks (group H1) or twice a day for the last 2 weeks (group H2) of a 4-week DMN treatment. The total amount of rh-HGF administered throughout the experimental period was equivalent between both groups. Administration of rh-HGF stimulated liver regeneration and ameliorated hepatic fibrosis, and treatment with a lower dose for a longer period of time (group H1) was more effective at inhibiting fibrosis than a shorter therapeutic course with a higher dose (group H2). HGF decreases both extracellular matrix (ECM) deposition and mortality in various models of liver cirrhosis in rats (21,22,24,25), and hepatic expression of TGF- $\beta$ , a key mediator of the development of liver cirrhosis, is suppressed by HGF treatment (21,23). We examined hepatic TGF- $\beta$  levels but, although a trend toward decreased TGF- $\beta$  levels was seen in animals treated with rh-HGF, our results were not statistically significant. In contrast, HGF apparently enhances TGF- $\beta$  production and DNA synthesis in activated c-Met expressing hepatic stellate cells (HSCs) isolated from rats treated with carbon tetrachloride (39). These seemingly contradictory effects of HGF on TGF- $\beta$  production may be explained by *in vitro* or *in vivo* experiments and/or differences in animal models of liver cirrhosis.

Hepatic fibrosis is a long-term consequence of chronic or repeated liver injury. Two different cell populations play a key role in the development of liver cirrhosis. Once hepatic injury occurs, quiescent HSCs are activated and undergo phenotypic changes, developing into myofibroblast-like cells

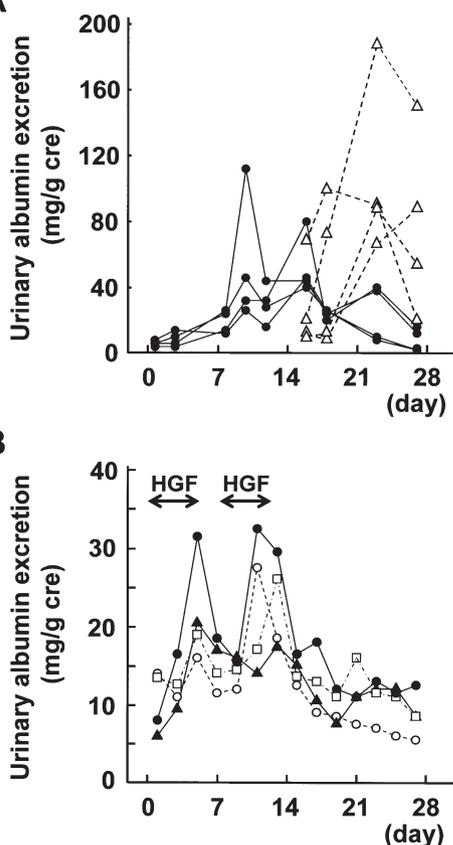


Figure 2. Sequential changes in urinary excretion of albumin in cirrhotic or normal rats treated with repeated intravenous injection of rh-HGF. (A) Administration of rh-HGF induced an increase in albuminuria in rats with DMN-induced liver cirrhosis. •, group H1 (n=4); △, group H2. (B) One mg/kg of rh-HGF was intravenously injected into normal rats (n=4) for days 1-5 and 8-12. Administration of rh-HGF increased urinary excretion of albumin. However, rh-HGF-induced albuminuria was a reversible change, which recovered after cessation of rh-HGF.

able to produce ECM proteins (40,41). In addition to activated HSCs, portal myofibroblasts also express ECM proteins involved in fibrotic changes of the liver (42,43). HGF stimulates the expression of matrix metalloproteinase-1 in human HSCs (44), and HGF inhibits cell growth and induces apoptosis in liver myofibroblasts, leading to the amelioration of hepatic fibrosis in cirrhotic rats (45). In the present study, we observed a more pronounced amelioration of increases in serum ALT levels in 2-week-treated rats (group H2) compared to 4-week-treated rats (group H1). Because the limited administration of HGF efficiently ameliorated experimental hepatic injury and rescued animals from lethal hepatic failure through anti-apoptotic effects (15-18), short-term administration and/or a high dose of rh-HGF appears sufficient to prevent tissue injury and resulting ALT release in DMN-induced cirrhotic rats. The ability of rh-HGF to prevent liver injury, maintain hepatic function and suppress the development of cirrhosis is probably mediated, at least in part, by the inhibition of HSC activation. Collectively, these data indicate that HGF prevents the development of cirrhosis through a variety of different mechanisms.

The liver is a primary target of intravenously administered rh-HGF in normal rats, and serum levels of rh-HGF were

elevated in rats with partial hepatectomy or a choline-deficient L-amino acid-defined diet compared to normal rats (26). These findings suggest that rh-HGF administered intravenously is primarily metabolized by the liver. In this study, we further investigated the pharmacokinetics of intravenously injected rh-HGF in rats with DMN-induced cirrhosis. Serum levels of rh-HGF 5 min after injection were elevated, and the CL was reduced, resulting in an increase in the  $AUC_{0-\infty}$  and  $MRT_{0-\infty}$  and a decrease in the  $V_{dss}$  in cirrhotic rats. HGF in the circulating plasma is efficiently extracted by the liver compared with other HGF target organs, and the liver is responsible for approximately 70% of the overall elimination of rh-HGF (46,47). Furthermore, HGF uptake is three times higher by hepatocytes than other liver non-parenchymal cells (48). Additionally, HGF receptor expression is saturable (48), and elimination of HGF from the systemic circulation is affected by changes in HGF receptor density on the liver cell surface (49). Therefore, a decrease in the number of hepatocytes and a change in c-Met expression in cirrhotic rat livers possibly affected the pharmacokinetics of rh-HGF. Also, the administered dose of rh-HGF (0.1 mg/kg) may be in excess leading to the saturation of receptor-mediated endocytosis and significant increases in serum concentration of rh-HGF.

When rh-HGF was injected intravenously as a bolus, the kidneys, spleen and adrenal glands as well as the liver contained large amounts of rh-HGF (26), and higher concentrations of rh-HGF in the systemic circulation increase the potential of adverse effects. In the present study, an increase in urinary albumin excretion was observed in cirrhotic rats treated with rh-HGF (0.3 mg/kg, once or twice a day). Although repeated doses of rh-HGF (1.0 mg/kg/day) increased urinary albumin excretion in normal rats, the effect was reversible, and treatment with rh-HGF did not affect serum creatinine levels, indicating the absence of permanent kidney injury. Further studies are needed to clarify the renal involvement following repeated doses of rh-HGF. However, since an increase in urinary albumin excretion is more sensitive to changes in kidney function than proteinuria (data not shown), the measurement of urinary albumin may be a useful marker for the detection of HGF-induced nephrotoxicity in clinical applications of rh-HGF.

In the present study, we showed that, although short-term administration of rh-HGF stimulated liver regeneration and ameliorated hepatic injury, prolonged treatment with rh-HGF was more effective in preventing cirrhosis development. However, elevated serum concentrations of rh-HGF in cirrhotic rats have the potential to increase the incidence of adverse events. Therefore, the development of a tissue-specific and/or sustained release delivery system is required for clinical applications of rh-HGF in the treatment of liver cirrhosis. The development of these technologies may also allow the application of rh-HGF in the treatment of diseases of extra-hepatic organs, such as inflammatory bowel disease, pulmonary fibrosis, or neurodegenerative disease.

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