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Continuous hepatocyte growth factor supply prevents lipopolysaccharide-induced liver injury in rats

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Abstract **We present a rat model in which continuous supply of hepatocyte growth factor (HGF) prevents liver injury induced by carbon tetrachloride (CCl_4) and *E. coli* 011:B4 lipopolysaccharide (LPS). Rat fibroblasts genetically modified to secrete rat HGF were implanted in syngenic rat spleen 7 days before administration of the hepatotoxins. Rats with HGF-secreting fibroblasts in the spleen showed a dramatic resistance to CCl_4- and LPS-induced liver injury. In the LPS-induced liver injury model, blood chemical analysis revealed that the increase in serum glutamic oxalacetic transaminase level and the decrease in blood sugar level were remarkably suppressed in rats with HGF-secreting cells in the spleen. Most importantly, their survival rate was greatly improved compared to other control groups of rats. Thus our results indicate a new role of HGF in liver protection during endotoxemia and convey important clinical implications for developing new therapeutic modalities in the treatment of liver failure caused by endotoxemia.**

Key words: Hepatocyte growth factor; CCl_4; Endotoxin; Liver injury; Protection

1. Introduction

Despite remarkable advances in chemotherapeutic and supporting agents, organ injury induced by septic syndrome due to Gram-negative bacteremia or endotoxemia is still one of the most threatening complications that follow trauma, burn and many types of surgery. The septic syndrome affects the lungs, liver, heart, kidneys, and gastrointestinal tract, frequently leading to multiple organ failure and produces high rates of morbidity and mortality [1,2]. Gram-negative bacterial lipopolysaccharide (LPS) is thought to mediate many pathophysiological manifestations of this syndrome. Although experimental animal models have provided the framework for the knowledge of the mechanisms of organ injury induced by LPS [3], an effective therapeutic approach remains to be established.

Development of cytokine therapy has greatly facilitated treatment of patients in various situations [4–8], while for organ injury induced by endotoxemia, successful treatment by cytokine has not been reported. Since most of the cytokines have rather short half-lives in vivo, continuous administration of recombinant products by use of pumps or repetitive injections have been employed to provide sustained serum level of a cytokine [9–11]. Another attractive way of continuous cytokine supply would be implantation of autologous or syngenic cells genetically modified to secrete recombinant product. Indeed, such cells have been shown to be of great advantage to therapy in animal models. Rosenthal et al. demonstrated that granulocyte-macrophage colony-stimulating factor-secreting, irradiated syngenic murine cells accelerate hematopoietic recovery after cytotoxic chemotherapy [12]. Recently, Moullier et al. reported that intraperitoneally implanted collagen lattices containing genetically modified autologous fibroblasts show a sustained secretion of human β-glucuronidase in dogs [13].

Hepatocyte growth factor (HGF) was originally found in the serum of partially hepatectomized rat [14] and in rat platelets [15] as the most potent stimulator of hepatocyte growth and DNA synthesis in vitro [16,17]. Recent reports showed that intravenous injection of purified human recombinant HGF (rHGF) enhanced liver or renal regeneration in mice [18,19], prevented acute renal failure [19] and suppressed the onset of liver cirrhosis induced by dimethylnitrosamine [20], suggesting that HGF may play important roles in the tissue repair process. We generated genetically modified syngenic cells to supply rHGF to the liver and demonstrate for the first time that continuous rHGF supply can successfully prevent LPS-induced liver injury in rats.

2. Materials and methods

2.1. Animals

Male F344 rats weighing 200–250 g (SRL, Hamamatsu, Japan) were used. All animals received humane care. A nutritionally balanced rodent diet and water were provided ad libitum.

2.2. Plasmids

Rat HGF cDNA [21] was inserted into the unique *Bam*HI site of the pCMV-Neo-Bam vector [22] to generate a rat HGF expression vector, pCMV/HGF.

2.3. Cells and transfection

One day before transfection, approximately 2×10^5 of Rat-1 cells [23], immortalized but not tumorigenic F344 rat-derived fibroblasts, were plated into a 6.0 cm dish with Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) containing 10% fetal calf serum (GIBCO, BRL, USA), penicillin (50 U/ml) and streptomycin (50 μg/ml) (standard medium). Rat-1 cells were transfected either with 2 μg of pCMV/HGF or 2 μg of pCMV-Neo-Bam plasmid DNA by the calcium phosphate method [24]. Transfectants were subjected to

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Abbreviations: rHGF, recombinant hepatocyte growth factor; LPS, lipopolysaccharide; GOT, glutamic oxalacetic transaminase; GPT, glutamic pyruvic transaminase; BS, blood sugar; CCl_4, carbon tetrachloride

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selection with 500 μg/ml of G418 (GIBCO, BRL, USA) and resultant G418-resistant colonies were either isolated, colony by colony, using a stainless-steel cylinder for rHGF-secreting cells or trypsinized and pooled for control cells.

2.4. ELISA assay and bioassay of rHGF

Conditioned medium derived from each established cell line was collected from confluent cultures. Portal blood was collected 7 days after cell implantation. HGF concentrations in conditioned medium or plasma from portal blood were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Institute of immunology, Tokyo, Japan). Biological activity of the rHGF was determined by BrdU staining of hepatocytes in primary culture as described previously [25].

2.5. Cell implantation

Rat-1/CMV cells or Rat-1/rat HGF3 cells ($\approx 3\times10^6$ cells) suspended in 0.1 ml of DMEM were injected into the spleen of F344 rats through a 26 G needle. The injection site on the surface of the spleen was immediately ligated to minimize leakage of implanted cells. To examine the deteriorative effects on the delivery of HGF by systemic circulation and the local connective tissue, we also implanted Rat-1/rat HGF3 cells ($\approx 6\times10^6$ cells) into the subcutaneous tissue of the upper back of rats.

2.6. Assessment of liver injury

Seven days after fibroblast implantation, CCl_4 (Wako, Osaka, Japan) dissolved in olive oil was intraperitoneally injected at a dose of 1.5 ml/kg body weight. Forty-eight hours after CCl_4 injection, the animals were killed and blood was collected from the aorta. Immediately after blood collection, each liver was removed for histochemical study (hematoxylin and eosin staining; HE staining) to observe histological change. To evaluate quantitatively the degree of liver injury after CCl_4 administration, the levels of serum glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were measured with an autoanalyzer. LPS from *E. coli* 011:B4 (DIFCO, Detroit, MI) (16 mg/kg body weight) dissolved in saline was intravenously injected 7 days after cell implantation. The animals were killed 6 h after LPS injection to evaluate the degree of liver injury in acute endotoxemia [26]. Rats were also examined before LPS treatment. The amounts of serum GOT, GPT, and blood sugar (BS) were measured with an autoanalyzer. The survival of rats was determined 48 h after LPS injection.

2.7. Statistical evaluation

Results are expressed as mean ± SD. Student's *t*-test was used to compare the mean values of the groups.

3. Results

3.1. Establishment of cell lines stably secreting rat rHGF

We established clonal cell lines secreting rat rHGF by transfection of Rat-1 cells with a HGF-expression vector, whose HGF production was determined by ELISA. As a control, Rat-1 cells transfected with the vector plasmid alone, selected and pooled were designated as Rat-1/CMV cells. HGF was barely detectable in conditioned medium prepared from control cells, while the clonal cell lines were found to produce large amounts of HGF. One of the established cell clones, Rat-1/rat HGF3 cell line was used in the following experiments, which produces the largest amount of rHGF at a rate of approximately 75 ng per 10^6 cells per 24 h. The secreted rHGF was confirmed to be biologically active on rat hepatocytes in primary culture. The labeling index of hepatocytes alone, hepatocytes provided with conditioned medium from Rat-1/CMV cells or Rat-1/rat HGF3 cells was 0.6%, 0.8% and 23.8%, respectively, indicating that rHGF produced by Rat-1/rat HGF3 cells serves as a biologically potent mitogen for rat hepatocytes in primary culture.

Table 1
HGF concentration in the portal blood was determined by ELISA assay 7 days after cell implantation

Group		HGF concentration (ng/ml)
Rat-1/CMV	(spleen)	<0.1
Rat-1/rat HGF3	(spleen)	$2.0\pm0.8^*$
Rat-1/rat HGF3	(subcutaneous)	<0.1

$^*P<0.001$ vs. control.

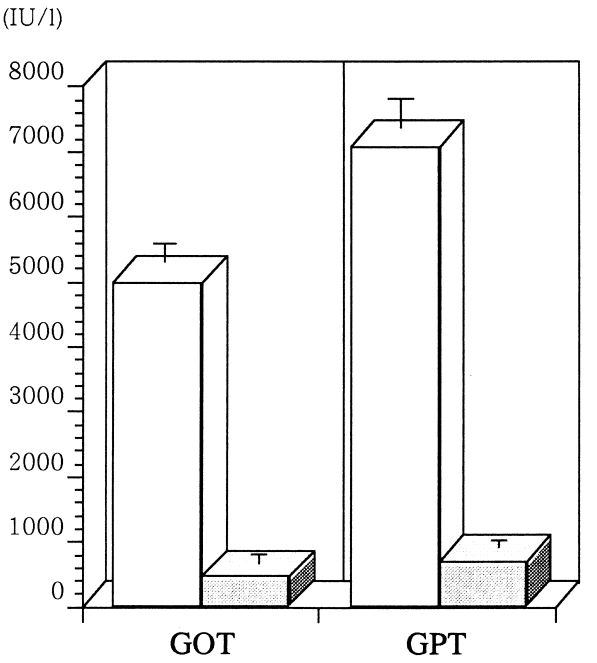

Fig. 1. Serum GOT and GPT levels 48 h after CCl_4 injection in rats implanted with Rat-1/CMV cells (white bars) or with Rat-1/rat HGF3 cells in the spleen (shaded bars).

3.2. Effects of rHGF on CCl_4-induced acute liver injury

To confirm the biological activity of secreted rat rHGF in vivo, we tested its protective effect on CCl_4-induced hepatocyte injury in the rat model we previously reported for human rHGF. Production of rHGF by the implanted cells was verified by measuring the concentration of HGF in the portal blood (Table 1). The mean HGF concentration in the portal blood 7 days after cell implantation, when implanted cells are found to secret rHGF stably, was 2.0 ± 0.8 ng/ml in rats with Rat-1/rat HGF3 cells in the spleen, while it was under detectable level (<0.1 ng/ml) in rats with Rat-1/CMV cells. Histological examination at 48 h after CCl_4 injection revealed that hepatocytes around the central veins were necrotic in rats implanted with Rat-1/CMV cells. However, necrosis was barely detectable in the liver of rats with Rat-1/rat HGF3 cells in the spleen. Fig. 1 shows that these histological findings correlated well with biochemical data that the increase in serum GOT and GPT levels caused by CCl_4 administration was markedly suppressed by implantation of Rat-1/rat HGF3 cells in the spleen ($P<0.001$). As compared with our earlier results with human rHGF [25], syngenic rHGF has proved to be more effective probably because of the absence of neutralizing antibody production in vivo.

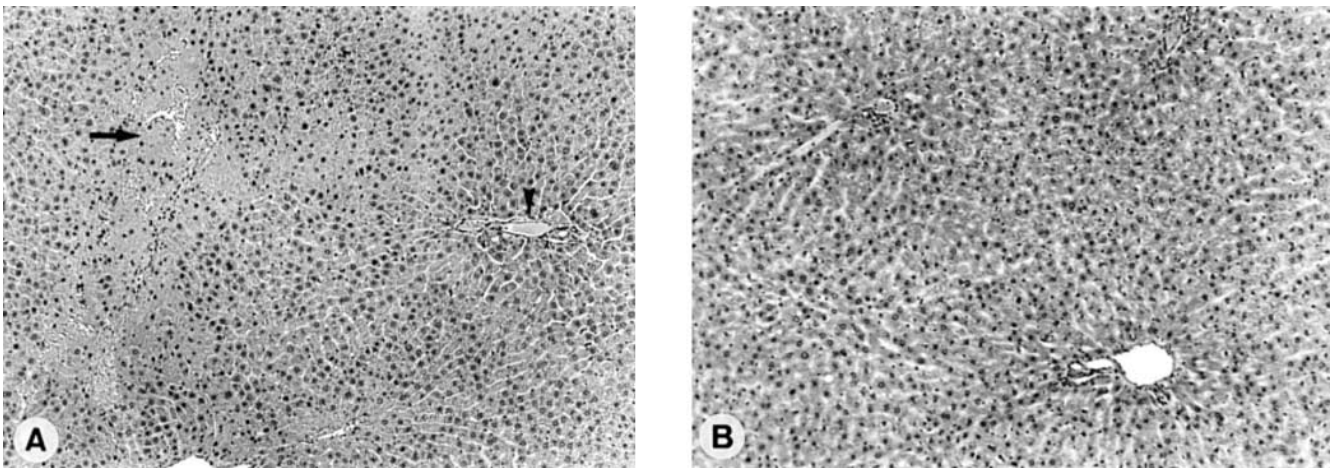

Fig. 2. HE staining of rat liver 6 h after LPS injection from rats implanted with Rat-1/CMV cells (A) or with Rat-1/rat HGF3 cells (B) in the spleen (×350). The arrow and arrowhead in (A) indicate massive hepatocellular necrosis and fibrin deposition in portal vein, respectively.

3.3. Implantation of Rat-1/rat HGF3 cells in the spleen prevents LPS-induced liver injury

We next analyzed the effect of rHGF supply on LPS-induced liver injury in the same rat model. Liver specimens taken from control rats 6 h after LPS challenge exhibited massive hepatocellular necrosis and fibrin deposition in portal veins, which were blocked almost completely by rHGF treatment (Fig. 2). The levels of GOT, GPT, and BS before LPS injection in rats implanted with Rat-1/rat HGF3 cells were not significantly different from those in control rats with Rat-1/CMV cells (Fig. 3). Six hours after LPS injection, the increase in serum GOT and GPT level was much reduced in rats with Rat-1/rat HGF3 cells ($54 \pm 7 \rightarrow 486 \pm 132$ IU/l, $48 \pm 8 \rightarrow 303 \pm 124$ IU/l, respectively) compared with that in control rats ($53 \pm 6 \rightarrow 1809 \pm 339$ IU/l, $46 \pm 7 \rightarrow 1382 \pm 229$ IU/l, respectively) ($P < 0.001$). Similarly, severe hypoglycemia observed in control rats ($167 \pm 23 \rightarrow 72 \pm 8$ mg/dl) was virtually prevented in rats with Rat-1/rat HGF3 cells in the spleen ($170 \pm 18 \rightarrow 127 \pm 13$ mg/dl) ($P < 0.001$). Implantation of Rat-1/rat HGF3 cells in the truncal subcutaneous tissue did not prevent the increase in the transaminase levels nor the severe hypoglycemia during acute endotoxemia. This can be explained by the fact that the mean HGF concentration in the portal blood 7 days after subcutaneous cell implantation was under detectable level (< 0.1 ng/ml) (Table 1). Importantly, at 48 h after LPS injection, 8 of 10 rats with Rat-1/rat HGF3 cells in the spleen survived whereas 2 of 10 rats with Rat-1/CMV cells in the spleen and 1 of 5 rats with Rat-1/rat HGF3 cells in the subcutaneous tissue were alive, indicating a crucial role of rHGF provided from fibroblasts implanted in the spleen (Table 2). In each group, most of the rats which could not survive were dead within 12 h after LPS injection, suggesting that the direct cause of the death is more likely LPS-induced endotoxin shock rather than liver failure.

Table 2
HGF supply from the spleen improves survival of rats in acute endotoxemia

Group		Survival rate	P value (vs. Rat-1/CMV)
Rat-1/CMV	(spleen)	2/10 (20%)	–
Rat-1/rat HGF3	(spleen)	8/10 (80%)	< 0.01
Rat-1/rat HGF3	(subcutaneous)	1/5 (20%)	NS

Seven days after fibroblast implantation, LPS from *E. coli* 011:B4 (DIFCO, Detroit, MI) (16 mg/kg body weight) dissolved in saline was intravenously injected. The survival of rats was determined 48 h after LPS injection.
NS: not significant.

4. Discussion

This report demonstrates that continuous and direct supply of rHGF to the liver represents a successful approach for prevention of liver failure caused by endotoxemia. The liver is a major organ for LPS detoxification during endotoxemia [27]. This process is accompanied by hepatocellular damage being initiated by the LPS-induced activation of phagocytic cells such as polymorphonuclear neutrophils and Kupffer cells which produce excess inflammatory cytokines and toxic chemical mediators including tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) [28,29]. In our model, however, it is not likely that rHGF directly modulated the activity of the hepatic macrophages since they are reported to lack HGF receptor expression [30]. While it has been reported that activated hepatic macrophages or neutrophils are mainly responsible for LPS-induced hepatocyte injury in vivo [3,29], CCl_4 is reported not to primarily activate phagocytic cells in the liver, but rather directly damage hepatocytes by oxidative stress [31]. Although recent reports have suggested possible involvement of TNF-α in the pathogenesis of CCl_4-induced hepatocyte necrosis [32,33], it is likely that the effects of hepatoxins on RNA and protein synthesis render normal hepatocytes susceptible to additional cytotoxicity from TNF-α as mentioned by Czaja et al. [32]. Thus HGF protects the liver from the two different types of injury, suggesting that it eventually protects hepatocytes directly or by interfering with the processes common to these types of liver injury.

On the other hand, since HGF affects a variety of epithelial and endothelial cells in their behavior, it is reasonable to assume that the exogenous HGF might have contributed to maintaining fundamental functions of other vital organs such as the lungs and heart during acute endotoxemia. The fact that most of rats which could not survive 48 h after LPS challenge were dead within early time points suggests that the direct cause of death might be rather LPS-induced endo-

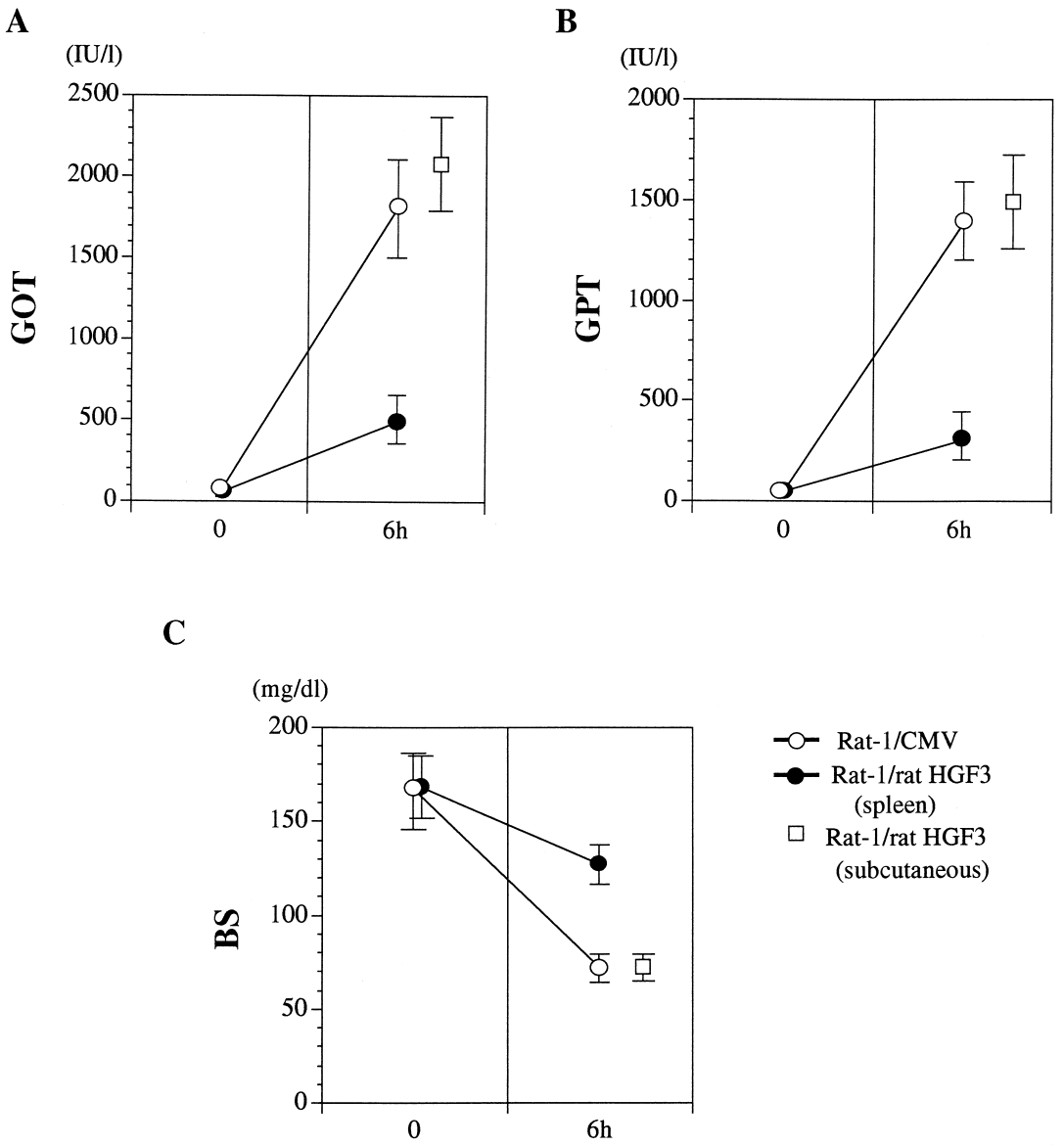

Fig. 3. The effects of HGF secreted from implanted fibroblasts on LPS-induced endotoxin shock. Serum GOT (A), GPT (B), and BS (C) levels of rats implanted with Rat-1/CMV cells (○) or with Rat-1/rat HGF3 cells in the spleen (●) were measured before or 6 h after LPS injection. Those in rats implanted with Rat-1/rat HGF3 cells in the subcutaneous tissue (□) were measured 6 h after LPS injection.

toxin shock than liver failure. We suppose that HGF might have not only cytoprotective effect for the liver but also anti-shock ability. We are currently investigating the effects of the rHGF on general circulation, renal and pulmonary functions as well as on hepatic sinusoidal cells in the same model.

A variety of animal models have been reported for the treatment of acute endotoxemia [34–38], most of which have focused on targeting bacterial products or suppression of cascades of immunologic and inflammatory events. However, attempts to protect targeted host organs from tissue injury during endotoxemia seem not yet to have been made extensively. We employed a new method of HGF supply for the following reasons. Firstly, since purified recombinant rat HGF protein is not currently available, cells genetically modified to secrete rat rHGF were used in this study to avoid neutralizing antibody production. Secondly, HGF de novo synthesized in the spleen would hardly disperse or lose its activity before reaching the liver as we implanted Rat-1/rat HGF3 cells in the organ directly upstream of the liver. Thirdly, the spleen may provide a desirable environment for implanted Rat-1/rat HGF3 cells to supply HGF instantly as it is rich in blood vessels. It awaits further investigation how rHGF affects the usual cytokine production in the spleen. Finally, as is thought to be the most important advantage, HGF could be continuously supplied throughout the entire course of the experiment. It is reported that the half-life time for HGF administered intravenously is less than 5 min [39]. While CCl_4 administration is known to cause liver injury as well as subsequent elevation of plasma HGF concentration over 100 ng/ml [40] which by far exceeds that of HGF presented in our model, we suppose that it is rHGF continuously supplied from the spleen that might keep targeted cells, including hepatocytes, in a primed state resulting in marked resistance against the hepatotoxins.

Our results would convey important clinical implications. Firstly, the spleen represents a suitable organ where genetically modified cells are implanted to provide recombinant products directly to the liver. Secondly, if one should view surgery as a 'planned wounding', it is tempting to speculate that pre-operative HGF supply could prevent liver failure caused by severe endotoxemia after major surgery including extended hepatectomy. Finally, given the role of HGF in promoting the integrity of hepatocytes as well as their survival, its continuous supply might ameliorate or arrest progression of chronic hepatic disorders. Thus, continuous delivery of rHGF would have a great deal of potential for prevention and treatment of liver diseases.

In conclusion, we presented clear evidences of successful liver protection from LPS-induced injury by rHGF although its clinical application requires further development of methods for practical supply of this cytokine.

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