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Exogenously Administered HGF Activator Augments Liver Regeneration through the Production of Biologically Active HGF

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Hepatocyte growth factor (HGF) plays a crucial role in the recovery of injured liver. Liver functions are mostly impaired in patients with liver diseases including cirrhosis. However, a significant amount of inactive HGF precursor (proHGF) is reported in the plasma of these patients. proHGF is proteolytically converted to an active form (mature HGF) by HGF-activator. Thus conversion of proHGF into mature HGF presumably contributes to the recovery of liver functions. In this study, rats with a partial hepatectomy were used, as proHGF is accumulated in the remnant liver. Recombinant human HGF-activator was administered via the portal vein to investigate the effect on molecular forms of HGF and its biological signaling. rhHGF-activator promptly converted pro-HGF into mature HGF, reaching maximal levels at 5–10 min after the injection, while the decreased pro-HGF was quickly recovered to the initial levels in the liver. The HGF receptor/c-Met was found to be autophosphorylated in the liver treated with rhHGF-activator. Further, the proliferating cell nuclear antigen labeling index and the liver regeneration rate were significantly higher in rhHGF-activator group than in control animals. These results indicate that exogenously administered HGF-activator produces a biologically active HGF from its precursor form and increases the potential for liver regeneration *in vivo*. © 2002 Elsevier Science

Key Words: **hepatocyte growth factor (HGF); HGF precursor; HGF-activator; HGF receptor/c-Met; partial hepatectomy.**

In the liver, hepatocyte growth factor (HGF) is synthesized and secreted by nonparenchymal liver cells including Kupffer cells in an inactive single-chain precursor form (proHGF) (1, 2), having a molecular weight of 92 kDa in humans and rats (3). The active mature HGF is produced from proHGF by proteolytic processing. Mature HGF is a heterodimeric form consisting of a heavy chain (α-chain, 62 kDa) and a light chain (β-chain, 32–36 kDa) held together by a disulfide bond. The heterodimeric form is required for the biological activities of HGF (3, 4), which are mediated through the stimulation of tyrosine kinase activity of its specific receptor encoded by c-Met protooncogene (5, 6) on the surface of hepatocytes.

Among the proteases reported to activate HGF such as urokinase- and tissue-type plasminogen activators (7, 8) and HGF-activator (9), we have found that HGF-activator cleaved proHGF to mature HGF *in vitro* with the most efficiency (10) and participated in tissue regeneration following hepatic and renal injury *in vivo* (11). It suggests that HGF-activator is a key enzyme regulating the activity of HGF in injured tissues. Stolz *et al.* (12) reported that urokinase-type plasminogen activator and its receptor also contributed to HGF-related signal transduction after partial hepatectomy. HGF-activator is a blood coagulation factor XII-like serine protease. HGF-activator precursor (96 kDa) is synthesized and secreted by parenchymal liver cells (9, 13), and circulates in the plasma, whereas the active form (34 kDa) is found in the serum. Thrombin is likely candidate for initiating the serum activation of HGF-activator precursor (14).

HGF acts predominantly as a mitogen on a wide variety of epithelial cells (15). In the liver, recent experimental and clinical evidence indicates that HGF plays a crucial role in liver regeneration (16–22). Liver regeneration after its resection is poor in patients with

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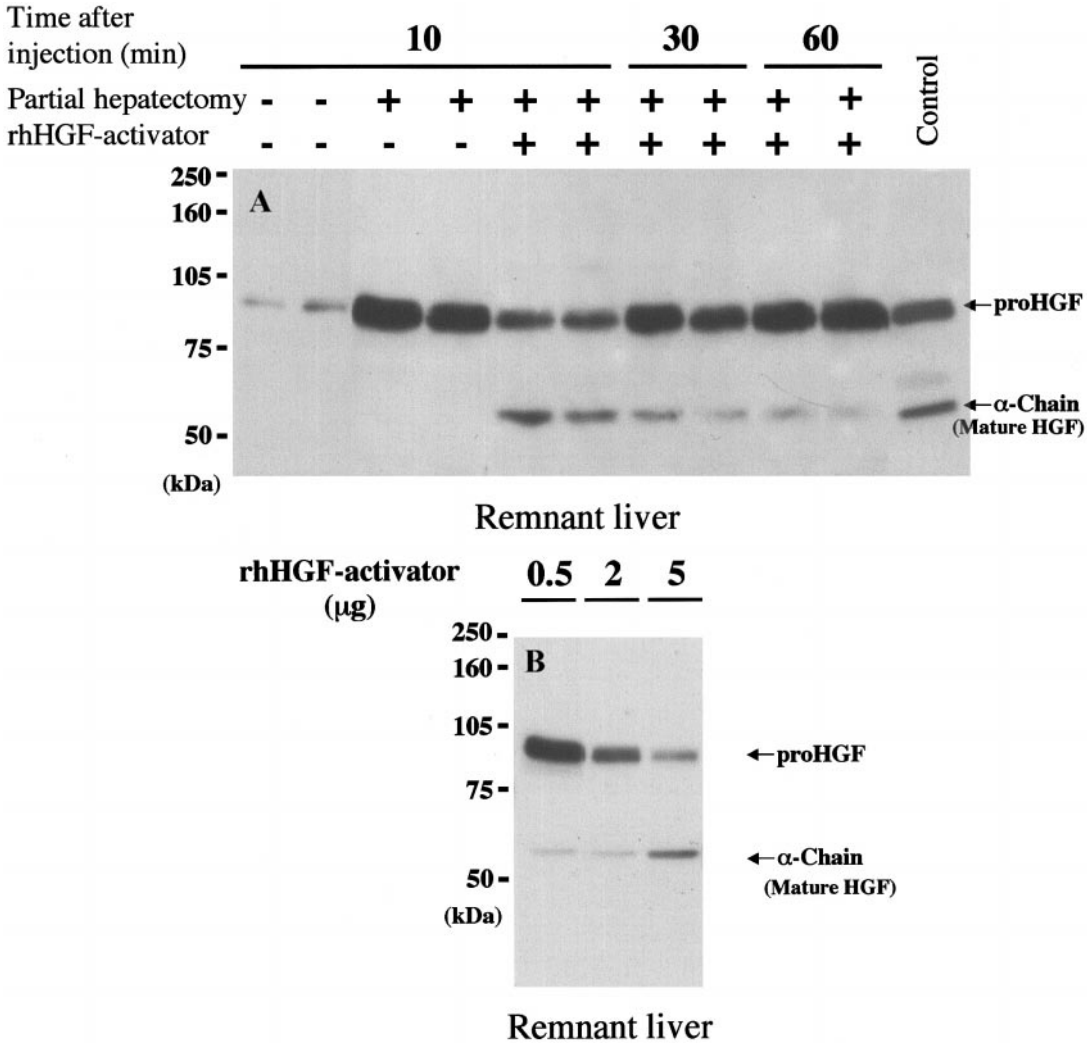

FIG. 1. The effect of rhHGF-activator on the molecular forms of HGF in partially hepatectomized rats. (A) The time course of proHGF conversion to mature HGF after the injection of rhHGF-activator into the liver. rhHGF-activator (5 μg protein) or its vehicle (saline) was administered via the portal vein at 24 h after a partial hepatectomy or a sham-operation. Then, crude HGF samples were prepared from the remnant liver at the indicated times and analyzed by immunoblotting (each sample from 0.5 g of liver/lane). As positive control, crude HGF sample prepared from the remnant liver at 24 h after the hepatectomy (0 time) was incubated *in vitro* with rhHGF-activator (2 ng protein) for 30 min at room temperature and used. (B) Dose dependency of the effect of rhHGF-activator. Crude HGF samples were prepared from the remnant liver at 10 min after the injection of rhHGF-activator (0.5, 2, and 5 μg protein/injection) in partially hepatectomized rats and analyzed. Data represent one of three independent experiments yielding similar results. α-Chain; a heavy chain (62 kDa) of mature HGF.

cirrhosis, since hepatic function is usually impaired before surgery in patients with cirrhosis. Levels of serum HGF in patients with liver diseases including cirrhosis were reported to be higher than those in the normal controls (23). Arakaki *et al.* (24) reported that a significant amount of proHGF was present in the plasma of patients with various liver diseases. Furthermore, an increase of HGF receptor/c-met mRNA expression was detected in hepatic tissues in patients with hepatitis, hepatocellular carcinoma and cirrhosis as compared with normal controls (25). Coexpression of c-met and HGF protein was observed in patients with liver cirrhosis (26).

These observations indicate that impairment of liver regeneration in liver diseases is in part because of the absence of proteases that convert proHGF to mature HGF, since it seems that levels of proHGF and HGF receptor/c-met are enough for the transduction of HGF signal. The conversion of endogenous proHGF into mature HGF may contribute the signaling involved in the regeneration and recovery of damaged tissues. We have found that HGF is markedly enhanced in the remnant liver after a 70% partial hepatectomy and persists as proHGF form (27). By using rats with the hepatectomy as experimental model, we investigate whether the injection of recombinant human HGF-

activator leads to the activation of proHGF *in vivo*, and, if so, whether the production of mature HGF functions as a mitogen and contributes to augmentation of liver regeneration in the remnant liver.

MATERIALS AND METHODS

Materials. Trasylol (trypsin-like protease inhibitor) was purchased from Bayer, Leverkusen, Germany. Phenymethylsulfonyl fluoride (PMSF) was from Sigma, St. Louis, MO. 6-Amidino-2-naphthyl-*p*-guanidinobenzoate dimethansulfate (nafamostat mesilate, protease inhibitor) was from Torii Pharmaceutical Co., Tokyo, Japan. 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS) was from Wako Pure Chemicals, Tokyo, Japan. SP-Sepharose was from Pharmacia LKB Biotech., Uppsala, Sweden. Centricon30 was from Millipore Corp. (Bedford, MA). All other chemicals were of reagent grade.

Partial hepatectomy and administration of rhHGF-activator. Sprague–Dawley rats (250–300 g) were hepatectomized by the method of Higgins and Anderson (28). 24 h after a 70% hepatectomy or sham-operation, rats were anesthetized and rhHGF activator (0.5–5 μg protein/ml of saline/rat) or its vehicle was administered via the portal vein following a midline laparotomy. In experiment with a clamp occlusion, the distal portion of the portal vein was occluded with a microvascular clamp just before injection of rhHGF-activator and the clamp was removed at 9 min after the injection. As a positive control for Western blot analysis (Fig. 2), normal rats were treated with a single intragastric dose of 0.4 ml of 50% CCl_4 in olive oil/100 g body weight, and liver and plasma samples were prepared after 24 h. Both of proHGF and mature HGF were found in the liver and plasma in CCl_4-treated rats (Figs. 2A and 2B; CCl_4) as reported previously (3, 27). The band corresponding to HGF-activator (34 kDa) was found in the plasma (Fig. 2C; CCl_4), which was presumably derived from endogenous HGF-activator precursor (94 kDa) existed in the plasma.

Preparation of recombinant human HGF-activator (two-chain form, 34 kDa). A cDNA encoding whole coding region of human HGF-activator (9) was subcloned into pcDNA3.1 vector (Invitrogen, De Schelp, NV Leek, The Netherlands). The plasmid was transfected into Chinese hamster ovary (CHO) cells, which were cultured in ERDF medium (Kyokuto Pharmaceutical Co., Tokyo, Japan) containing 0.4 mg/ml G418 and 5% fetal calf serum (FCS). G418-resistant colonies were selected and screened for the expression of HGF-activator using an enzyme-liked immunosorbent assay (29). CHO cells expressing HGF-activator were cultured in ERDF containing 100 μM nafamostat mesilate, 0.4 mg/ml G418, and 5% FCS for 10 days. The conditioned medium was applied to Sulfate-Cellulofine column (Chisso Corp., Tokyo, Japan) and then to A6 mAb affinity column (14). The purified single-chain 96 kDa HGF-activator (inactive precursor) was activated to 34 kDa two-chain form by treatment with plasma kallikrein and thrombin. The two-chain 34 kDa rhHGF-activator was purified using an A6 mAb affinity column.

Preparation of crude HGF from the liver and plasma. The liver was perfused with saline, excised and homogenized in 4 vol of 50 mM Tris–HCl buffer, pH 8.5, containing 10 mM EDTA, 1 mM PMSF, 100 μM nafamostat mesilate and 0.15 M NaCl or immediately frozen in liquid nitrogen and stored at −80°C until use. Crude HGF was prepared according to the method of Miyazawa *et al.* (3, 27). Blood samples (6 ml) were collected from the bifurcation of the abdominal aorta with an anticoagulant (0.84 ml; 3.2% trisodium citrate containing 200 U/ml Trasylol). After centrifugation (1600*g* for 10 min), the supernatant (plasma, 2 ml) was mixed with 4 vol of 20 mM Tris–HCl buffer, pH 7.4, containing 200 U/ml Trasylol, 100 μM nafamostat mesilate, 0.1% CHAPS and 0.4 M NaCl (solution B), and applied to SP-Sepharose column. The eluate from SP-Sepharose column was concentrated by ultrafiltration (Centricon30) and used for Western blot analysis. proHGF and mature HGF were distinguished by immunoblotting under reduced conditions with mouse monoclonal antibodies against a peptide sequence in the heavy (α-) chain of human HGF (30).

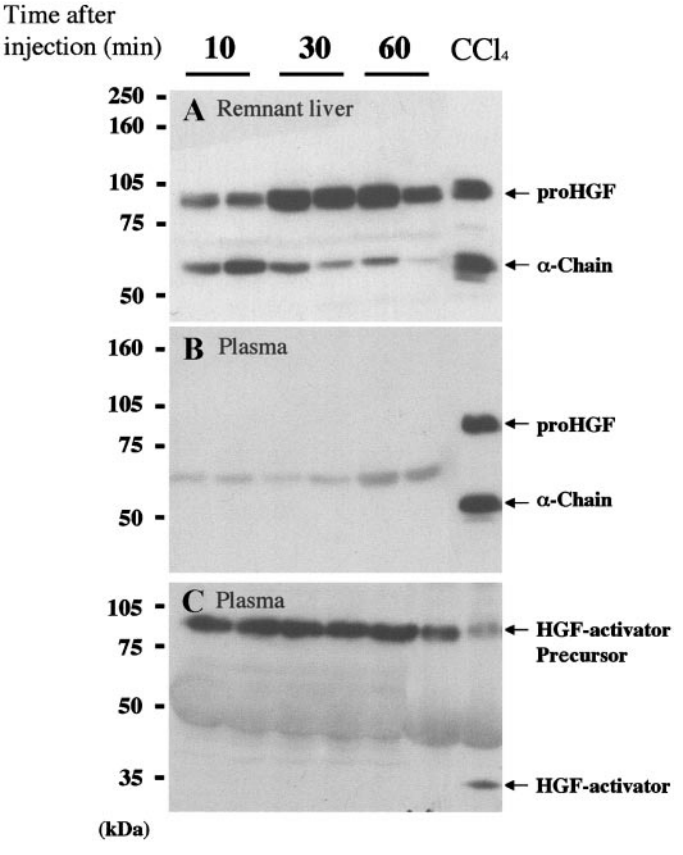

FIG. 2. The effect of rhHGF-activator on the molecular forms of HGF under the conditions with a clamp occlusion. rhHGF-activator was administered in partially hepatectomized rats as described in the legend to Fig. 1A, except that the distal portion of portal vein was occluded with a clamp before the injection, followed by declamping at 9 min after the injection. Crude HGF samples were prepared at the indicated times from (A) the remnant liver (0.5 g) and (B) plasma (0.5 ml) and analyzed. The band, which is found at 50–75 kDa, in the plasma (B) is nonspecific one. (C) HGF-activator and its precursor in the plasma (2 μl) were analyzed. As positive control (CCl_4), crude HGF was prepared from the liver (0.5 g) and plasma (0.5 ml) of CCl_4-treated rats, and the plasma sample (2 μl) was used for analysis of HGF-activator. Data represent one of three or four independent experiments yielding similar results.

Immunoprecipitation (IPP) of the HGF receptor/c-Met in the remnant liver. A modified method of Stolz *et al.* (12) was used for precipitation. Frozen liver (0.35 g) was homogenized in 2 ml of lysis buffer (10 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA, 1 mM sodium orthovanadate, 0.2 mM PMSF, 500 U/ml Trasylol and 100 μM nafamostat mesilate). The homogenate (900 μl) was mixed with 100 μl of 10% sodium dodecyl sulfate (SDS), sonicated and left to stand for 1 h. Liver lysate (5 mg of protein/200 μl) was mixed with 1800 μl of IPP solution (10 mM Tris–HCl buffer, pH 7.4, containing 1% Triton X-100, 0.5% Nonident P-40, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM PMSF and 100 U/ml Trasylol). The supernatant after precleared with protein A-insoluble (Sigma) was incubated with antibody (2 μg/ml) against mouse c-Met (rabbit polyclonal m-Met; SP260, Santa Cruz) overnight, followed by addi-

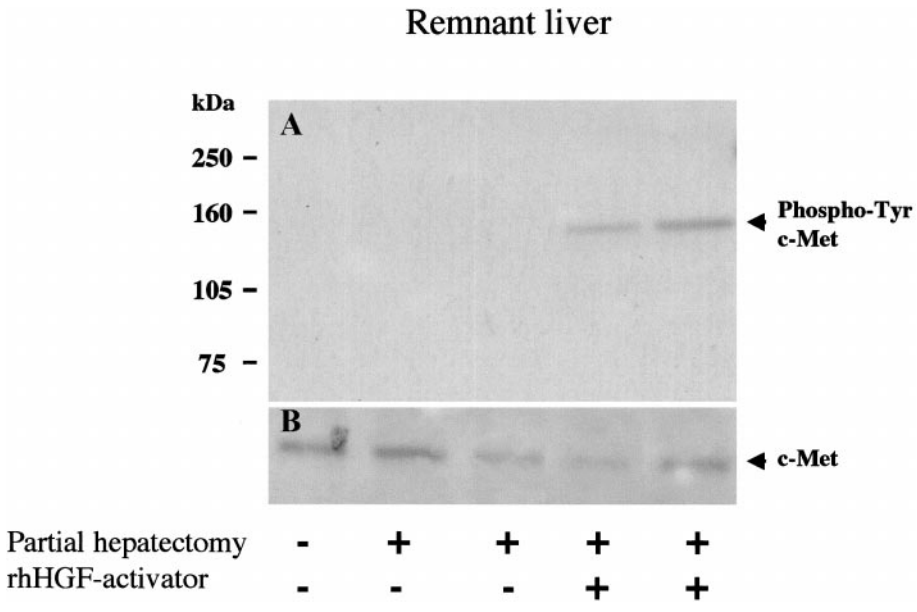

FIG. 3. The effect of rhHGF-activator on the activation of HGF receptor/c-Met. Rats were treated for 10 min with rhHGF-activator (5 μg protein) or its vehicle in partially hepatectomized or sham-operated rats. Liver lysates obtained (corresponding to 2 mg protein) were coimmunoprecipitated with rabbit polyclonal antibody against mouse c-Met and protein A–Sepharose. Immunoprecipitated proteins were resolved on 7.5% SDS–PAGE gels, blotted to PVDF, and probed with (A) mouse monoclonal anti-phosphotyrosine antibody or (B) mouse monoclonal anti-mouse c-Met antibody. Data represent one of two independent experiments yielding similar results.

tion of protein A-Sepharose CL-4B (Pharmacia). The IPP pellet was used for Western blot analysis. The concentration of protein was determined using the DC protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as standard.

Western blot analysis. Samples were solubilized in 125 mM Tris–HCl buffer, pH 6.8, containing 5% glycerol, 2% SDS and 1% 2-mercaptoethanol, boiled at 100°C for 3 min and subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on 7.5% gels and electroblotted onto a polyvinylidene difluoride (PVDF; Bio-Rad, Hercules, CA) membrane. Immunostaining was performed using an ECL blotting detection agent (Amersham Co., Amersham, Bucks, UK) and mouse monoclonal antibodies against heavy (α-) chain of human HGF (30), human HGF-activator (A-1) (11), phosphotyrosine (4G10, Upstate Biotech, Lake Placid, NY) or mouse c-Met (m-Met (B2), Santa Cruz Biotech., Santa Cruz, CA) as the primary antibody.

Measurement of proliferating cell nuclear antigen (PCNA) labeling index and liver regeneration rate. Rats were decapitated at the indicated times after the 70% partial hepatectomy. PCNA is a nuclear protein that shows maximal levels in the S phase of proliferating cells, and there is a positive correlation between the PCNA labeling index and incorporation of thymidine or bromodeoxyuridine into DNA (31–33). To assess DNA synthesis by hepatocytes in the remnant liver after hepatectomy, the PCNA labeling index was measured as previously reported (34). The PCNA labeling index was determined by the random evaluation of 5–10 fields (at least 2000 hepatocytes). The index was expressed as the following percentage: (the number of cells with positive nuclei/total number of hepatocytes counted) $\times$ 100.

In preliminary experiment, the resected liver and the remaining liver were weighed in rats after the partial hepatectomy, and the percentage of resected liver weight relative to the original whole liver was 68.7 $\pm$ 5.6% (means $\pm$ SD, n = 10). Thus, the original whole liver weight was calculated as the resected liver weight $\times$ 100/68.7. When rats were sacrificed at the indicated times after the hepatectomy, the remnant liver was excised and weighed, and the liver regeneration rate (%) was expressed as follows: remnant liver weight/[original whole liver weight] $\times$ 100.

Statistics. Data were expressed as mean $\pm$ SD. Differences between groups were identified by the unpaired Student t test and $P < 0.05$ was taken to indicate statistical significance.

RESULTS

Injection of recombinant human HGF-activator via the portal vein in partially hepatectomized rats. HGF is expressed at low levels in the liver of normal rats as an inactive single-chain precursor form (proHGF, 92 kDa). The levels of HGF are markedly enhanced after a 70% partial hepatectomy, with maximal levels at 12–24 h after resection. The increased HGF is located predominantly in the remnant liver and persists as proHGF (27). We administered rhHGF-activator via the portal vein at 24 h after the partial hepatectomy. The injection of rhHGF-activator stimulated the conversion of proHGF to mature HGF in the remnant liver (Fig. 1A), which is a biologically active heterodimeric form with heavy (α-) and light (β-) chains. The α-chain of mature HGF appeared at maximal levels at 10 min after the injection of activator, and then decreased gradually. No difference in levels of mature HGF was detected between 5 and 10 min after the injection of rhHGF-activator (data not shown). Levels of proHGF markedly decreased at 10 min after the injection of activator, and were restored to initial levels at 30 min. The conversion of proHGF to mature HGF by rhHGF-activator was dose-dependent, showing a high efficiency at 5 μg/injection (Fig. 1B).

The short-term recovery of proHGF levels implied that rhHGF-activator is not retained within the liver but

passes into the blood circulation after its injection. We examined whether rhHGF-activator passes through the liver or not. In order to restrict the outflow of rhHGF-activator, we injected rhHGF-activator while a clamp occlusion was applied at the distal portion of portal vein. The clamp was then removed at 9 min after the injection. As shown in Fig. 2A, the time course of the production of mature HGF from proHGF under the clamped conditions was similar to that obtained without a clamp (Fig. 1A). Further, none of exogenously injected rhHGF-activator (34 kDa) was detected in the plasma (Fig. 2C). In the case of HGF, a tendency was observed that levels of mature HGF produced by rhHGF-activator were less than the decrease of proHGF, implying a loss of HGF from the liver. However, neither mature HGF nor proHGF was detected in the plasma (Fig. 2B). These results suggest that rhHGF-activator and mature HGF are not leaked from the liver.

Effect of mature HGF produced by rhHGF-activator on liver regeneration. A high-affinity HGF receptor, c-Met (140 kDa), has been identified in various tissues including liver parenchymal cells (hepatocytes) and has a tyrosine kinase domain that is autophosphorylated by mature HGF. Experiments with immunoprecipitation of HGF receptor/c-Met revealed that there was no tyrosine phosphorylation of c-Met in the remnant liver at 24 h after partial hepatectomy and the injection of rhHGF-activator markedly increased the tyrosine phosphorylation of c-Met (Fig. 3A). Levels of c-Met protein were unchanged in sham-operated, partially hepatectomized and partially hepatectomized/rhHGF-activator-treated groups (Fig. 3B). The tyrosine phosphorylation of c-Met indicates that intracellular signaling for HGF is transmitted through its receptor. In support of this observation, rhHGF-activator enhanced the DNA synthesis and the liver regeneration in the remnant liver. The administration of rhHGF-activator at 24 h (on day 1) after the hepatectomy resulted in a significantly increased PCNA labeling index on day 2 over that in rats injected with saline alone (Fig. 4A). The liver regeneration rate also increased in rats with rhHGF-activator (82.3 ± 7.6%, means ± SD; $n = 9$) as compared with controls (64.9 ± 9.2%; $n = 9$) on day 4 (Fig. 4B).

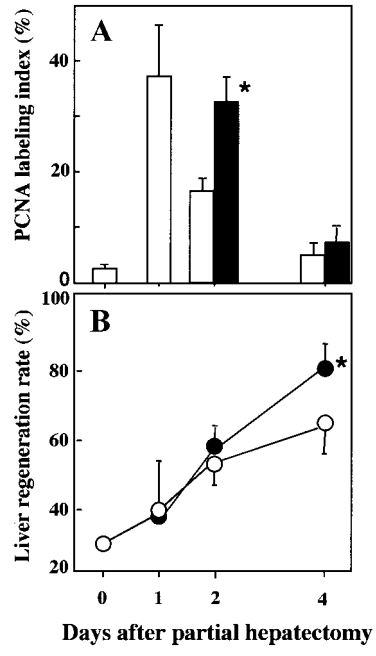

FIG. 4. The effect of rhHGF-activator on liver regeneration. rhHGF-activator (5 μg protein, closed) or its vehicle (open) was administered via the portal vein at 24 h after a partial hepatectomy. Livers were excised at the indicated times and were used for measurements of (A) PCNA labeling index and (B) liver regeneration rate. Data represent the mean ± SD ($n = 6$–9). *$P < 0.05$ vs without rhHGF-activator.

DISCUSSION

In this study, we found that the injection of rhHGF-activator via the portal vein converted the precursor form of HGF (proHGF) to the heterodimeric form of HGF (mature HGF) in partially hepatectomized rats (Fig. 1). The mature HGF reached maximal levels in the liver at 5–10 min after the injection of rhHGF-activator. Mature HGF produced by rhHGF-activator functioned as a mitogen through the tyrosine phosphorylation of c-Met (Fig. 3), which was followed by increases in the PCNA labeling index and liver regeneration rate (Fig. 4). This is the first report showing the production of biologically active HGF from endogenous proHGF by exogenously injected HGF-activator in the liver. proHGF as well as mature HGF is present in plasma of patients with fulminant hepatic failure, acute hepatitis, chronic hepatitis or cirrhosis (24), implying an existence of HGF precursor in the liver. Conversion of proHGF into mature HGF by rhHGF-activator may contribute to the enhancement of liver function and regeneration in patients with liver diseases.

Experiments with Western blot analysis revealed that mature HGF produced by rhHGF-activator was detected in the liver but not in the plasma (Fig. 2B). Mature HGF was probably retained in the remnant liver, since HGF binds to heparin and heparan sulfate proteoglycans (35, 36), which are located on cell surfaces and within the extracellular matrix. The rapid decrease of mature HGF may be due to internalization with its receptor, c-Met, followed by degradation (37). We also found that rhHGF-activator did not leak from the liver after injection (Fig. 2C). However, the decreased proHGF was recovered to the initial levels within 30 min, irrespective of clamping of the portal vein (Figs. 1A and 2A). Thus, it seems likely that the action of rhHGF-activator is transient and quickly inactivated in the liver. Recently, HGF-activator inhibitor type 1 (HAI-1) was identified as an integral mem-

brane and complexes with the active form of HGF-activator (29, 38). rhHGF-activator may interact with its inhibitor, HAI-1, located on the cell surface or extracellular matrix within the liver, resulting in the inactivation of its activity.

We previously reported that the continuous infusion of recombinant human HGF stimulated liver regeneration and function after a partial hepatectomy in cirrhotic rats (34). We found that a significant high level of recombinant human HGF was circulated in blood during the infusion periods (7 days), indicating that HGF acts on other tissues and organs in addition to the liver. Higher levels of HGF may promote tumorigenesis (39) and migration of human hepatocellular carcinoma (40). In contrast, the present work demonstrates that both rhHGF-activator and mature HGF remain and act transiently within the liver but are not available outside the liver. Thus, the injection of rhHGF-activator via the portal vein into the liver restricted its activity to the liver. In our preliminary experiment, rhHGF-activator was injected into the penile vein instead of the portal vein in partially hepatectomized rats. This administration of rhHGF-activator did not produce a detectable level of mature HGF in the liver and plasma, and also had no effect on the PCNA labeling index and the liver regeneration rate in the remnant liver. rhHGF-activator may be diluted and/or absorbed by heparin-like substances and its inhibitors in the blood circulation, and could not reach to the liver.

In conclusion, radical resection is accepted as one of the most curative treatments for hepatocellular carcinoma. However, most patients have coexisting cirrhosis and their liver function is usually impaired. Therefore, stimulation of functions including the regeneration of the remnant liver after surgery is important. The injection of exogenous rhHGF-activator may be potentially useful for the treatment of patients with hepatocellular carcinoma and liver cirrhosis after surgery.

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