

Activation of Hepatocyte Growth Factor in the Injured Tissues Is Mediated by Hepatocyte Growth Factor Activator*

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Hepatocyte growth factor (HGF) is a potent mitogen, motogen, and morphogen for epithelial cells *in vitro*. It appears likely that HGF participates in tissue regeneration following hepatic and renal injury *in vivo*. The activity of HGF is localized to the injured tissues by a proteolytic activation system; HGF remains as an inactive single-chain form in the normal state and is converted to an active heterodimeric form in response to tissue injury. A protease responsible for this conversion is induced in the injured liver, but it has not yet been identified. We have previously purified and characterized HGF activator (HGFA), a serum-derived serine protease that efficiently activates single-chain HGF *in vitro*. In this study, we found that the HGF-converting activity in the injured liver was inhibited by an anti-HGFA antibody. We also found that the active form of HGFA was generated exclusively in the injured tissues. Thus, it appears likely that HGFA is the key enzyme that regulates the activity of HGF in the injured tissues. We also analyzed the heparin binding properties of the precursor and mature forms of HGFA. HGFA had a weak affinity for heparin near the physiological salt concentration in its precursor form but acquired a strong affinity for heparin upon activation that is linked to blood coagulation. This property may ensure the local action of this enzyme at the site of tissue injury.

Hepatocyte growth factor (HGF)¹, also known as scatter factor, was originally described as a potent mitogen for hepatocytes in primary culture (1–3). It was subsequently shown to have mitogenic, motogenic, and morphogenic activities on various target cells, including renal tubular epithelial cells and vascular endothelial cells (4–9). HGF is thought to play an important role in regeneration following hepatic and renal injury (10–12).

Mature HGF is a heterodimeric protein consisting of a heavy chain and a light chain held together by a disulfide bond (1). The two chains are produced from a single-chain precursor by

proteolytic processing (13, 14). This processing, which is mediated by a serine protease, is required for HGF to exert both its mitogenic and motogenic activities (15–19). We recently found that the biological effects of HGF in injured tissues are regulated through this proteolytic processing; HGF in normal tissue is present in the inactive single-chain form, and it is converted to the active heterodimeric form exclusively in the injured tissues (20). We also found that this conversion was mediated by a serine protease, the activity of which was induced in the injured tissues (20). However, the serine protease has not yet been identified.

Four proteases are reported to activate HGF *in vitro*. We previously purified a HGF-activating protease from bovine and human serum (21, 22) and designated it HGF activator (HGFA). Blood coagulation factor XIIa, urokinase, and tissue-type plasminogen activator (tPA) also activate HGF *in vitro* (16, 23, 24). Although the action of urokinase and tPA on HGF is very weak *in vitro* (23, 25), it is possible that, *in vivo*, the enzymatic reaction may be stimulated by a cofactor(s) or by a certain microenvironment. In fact, receptor-bound urokinase modulates the activation and receptor binding of HGF (26). These serine proteases are thus the candidates for the HGF-converting enzyme(s) in injured tissues.

In the present study, we examined the involvement of HGFA in the activation of HGF in the injured tissues. We purified and characterized the rat counterpart of HGFA. We then analyzed the inhibitory effect of anti-human HGFA monoclonal antibodies (mAb) on rat HGFA and found that one of them inhibited rat HGFA. We demonstrated that the HGF-converting activity in the homogenate of injured rat liver was abrogated by treatment with the anti-HGFA antibody. In addition, we found that the active form of HGFA was generated exclusively in the injured tissues. Thus, we concluded that HGFA was the key enzyme regulating the activity of HGF in injured tissues. We also found that the activated HGFA acquired the capacity to interact with heparin. This property may ensure the local action of HGFA.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained as follows: 6-amidino-2-naphthyl-p-guanidinobenzoate dimethanesulfonate (nafamostat mesilate) from Torii Co. Ltd.; phenylmethylsulfonyl fluoride (PMSF) and CHAPS from Sigma; and SP-Sepharose Fast Flow, heparin-Sepharose CL-6B, Sepharose CL-6B, and CNBr-activated Sepharose 4B from Pharmacia Biotech Inc. For an affinity gel, 1 mg of P1–4 HGFA mAb was coupled to 1 ml of CNBr-activated Sepharose 4B. Single-chain HGF was prepared as described previously (21).

Rat HGF Activator—Rat HGFA was purified from rat serum (Chemicon International Inc.) by the same procedure as that described for bovine HGFA (21). HGFA activity was assayed in 10 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl and 0.01% CHAPS, as described previously (27). The effects of serine protease inhibitors on HGFA were examined as described previously (21).

Assay for HGF-converting Activity in Homogenate of Injured Liver—

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¹ The abbreviations used are: HGF, hepatocyte growth factor; HGFA, hepatocyte growth factor activator; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; tPA, tissue-type plasminogen activator.

Male rats of the Wistar strain (190–220 g) were intragastrically administered by 50% CCl₄ in olive oil (0.4 ml/100 g of body mass). After 8 h, the liver was removed and homogenized in 4 volumes of ice-cold buffer containing 20 mM HEPES, pH 7.3, 0.15 M NaCl, 10 mM EDTA, and 5 units/ml heparin. 0.5 ml of the homogenate was diluted to 1 ml with the homogenizing buffer and supplemented with CHAPS to 0.1%; a monoclonal antibody was added at 400 µg/ml. After gentle agitation at 4 °C for 24 h, 2 µg of single-chain HGF was added, and the reaction mixtures were incubated at 37 °C for 8 h. The reaction was terminated by the addition of 1 mM PMSF and 100 µM nafamostat mesilate, followed by centrifugation at 12,000 × *g* at 4 °C for 20 min. The resultant supernatant (500 µl) was diluted with 5 ml of 50 mM Tris-HCl, pH 8.5, containing 0.15 M NaCl, 10 mM EDTA, 1 mM PMSF, 100 µM nafamostat mesilate, and 0.1% CHAPS and applied to an SP-Sepharose column (0.65 × 0.8 cm). The HGF-rich fraction was eluted and analyzed by immunoblotting as described previously (20).

Analysis of Molecular Forms of Tissue-derived HGFA—Hepatic injury was induced as described above. Renal injury was induced by subcutaneous injection of 0.6 mg of HgCl₂/100 g of body mass (20). After 12 h, rats were decapitated, and the tissues were removed. Tissues from at least four animals were collected and homogenized in 4 volumes of ice-cold buffer containing 20 mM HEPES, pH 7.3, 0.15 M NaCl, 10 mM EDTA, 100 µM nafamostat mesilate, and 1 mM PMSF. NaCl concentration of the homogenate was adjusted to 0.65 M. After incubation for 30 min at 4 °C, the homogenate was centrifuged at 105,000 × *g* for 60 min at 4 °C. An aliquot of the supernatant containing 200–300 mg of protein was supplemented with CHAPS to 0.1% and applied to a P1–4 mAb affinity column (0.8 × 0.4 cm) pre-equilibrated with 20 mM HEPES, pH 7.3, containing 0.65 M NaCl, 10 mM EDTA, and 0.1% CHAPS. The column was washed with 4 ml of the same buffer. HGFA was eluted with 2 ml of 50 mM glycine HCl, pH 3.0, containing 0.1% CHAPS. The eluate was neutralized with 100 µl of 2 M Tris-HCl, pH 8.0, concentrated by ultrafiltration (Centricon 30, Amicon), resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (8.5% acrylamide) under reducing conditions, and analyzed by immunoblotting using an anti-HGFA monoclonal antibody A-1.

Heparin-Sepharose Chromatography of Rat Plasma and Serum—To prepare rat plasma, fresh rat blood (2 ml) was mixed with 0.25 ml of 77 mM EDTA and 0.25 ml of 5% glucose solution containing 1 mM nafamostat mesilate and 10 mM PMSF and was immediately centrifuged. Rat serum was prepared from the same animal and treated with 1 mM PMSF at 4 °C for 24 h before use. 200 µl of rat plasma or serum was diluted 3-fold with 10 mM phosphate buffer, pH 7.0, containing 0.05% CHAPS and 1 mM PMSF and applied to a heparin-Sepharose CL-6B column (0.8 × 0.4 cm; bed volume, 200 µl) equilibrated with 10 mM sodium phosphate, pH 7.0, containing 0.05% CHAPS and 50 mM NaCl. The column was washed with 4 ml of the equilibration buffer containing 1 mM PMSF. The bound proteins were then eluted in a stepwise manner with 1 ml each of 10 mM phosphate buffer, pH 7.0, containing 0.05% CHAPS and increasing concentrations of NaCl (100–800 mM). Each of the eluates was concentrated with a Centricon 30 (Amicon), resolved by SDS-PAGE (8.5% acrylamide) under reducing conditions, and then analyzed by immunoblotting, using an anti-HGFA antibody A-1.

RESULTS

Purification and Characterization of Rat HGF Activator—Rat HGFA was purified from rat serum by the same procedure as that described for the purification of bovine HGFA. The final preparation showed a single stainable band on SDS-PAGE under both reducing and non-reducing conditions. The apparent molecular masses were 33 kDa under reducing conditions and 29 kDa under non-reducing conditions. Twenty µg of purified protein converted recombinant human single-chain HGF at low doses, comparable with human HGFA (Fig. 1A). The sensitivity of rat HGFA to various serine protease inhibitors was the same as that of bovine and human HGFA (data not shown).

Inhibition of Rat HGFA by the P1–4 Monoclonal Antibody—We have recently obtained 8 mAb raised against human HGFA (23, 27). Here, we examined the effect of these antibodies on the action of purified rat HGFA (Fig. 1B). Of these 8 mAb, the P1–4 mAb most efficiently inhibited rat HGFA. P1–4 did not inhibit the HGF-converting activity of factor XIIa (23),

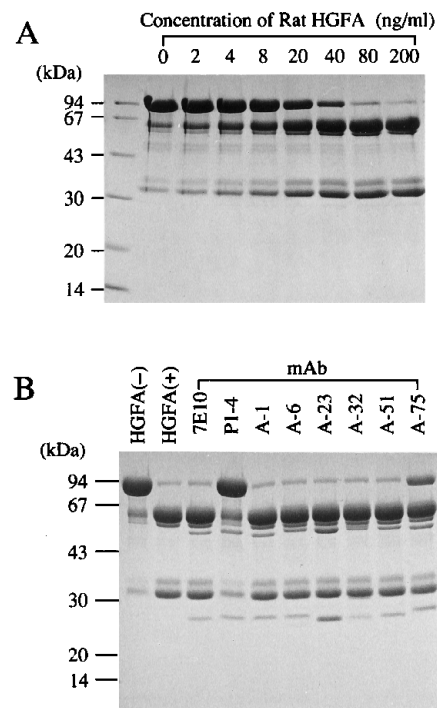


FIG. 1. Activation of human recombinant HGF by rat HGFA (A) and the effects of monoclonal antibodies (B). A, single-chain HGF (200 µg/ml) was incubated at 37 °C for 2 h with various concentrations of rat HGFA and analyzed by SDS-PAGE (12.5% acrylamide) under reducing conditions. The gel was stained with Coomassie Brilliant Blue. Molecular mass markers are shown on the left. B, effects of monoclonal antibodies (1 µg) were analyzed by preincubation with rat HGFA (10 ng) at 37 °C for 30 min.

and it also did not inhibit plasminogen activation by urokinase and tPA when analyzed by the fibrin plate method (28) (data not shown). The P1–4 mAb was therefore used to analyze HGF-converting activity in injured rat liver.

Inhibition of HGF-converting Activity in Injured Liver by the P1–4 Monoclonal Antibody—HGF-converting activity was induced in the liver after the rat was treated with CCl₄ (20). We examined the effect of the anti-HGFA antibody, P1–4, on this activity (Fig. 2). The P1–4 antibody inhibited more than 80% of the HGF-converting activity in the injured liver (lane 3), whereas the P-5 antibody, which does not react with HGFA, did not affect the activity (lane 4).

Presence of the Active Form of HGFA in Injured Tissues—HGFA is secreted as a 96-kDa inactive zymogen and requires proteolytic conversion to exert its activity (27). Thus, the active form is expected to be generated in injured tissues. We examined the form of HGFA in injured and uninjured tissues after induction of hepatic or renal injury (Fig. 3). The amount of HGFA was reduced in all tissues examined after induction of hepatic injury, and the reduction in the kidney was remarkable. The 33-kDa active form of HGFA was detected in the liver but not in the kidney, lung, and spleen after induction of hepatic injury (Fig. 3, lane 2). After induction of renal injury, the amount of HGFA increased in the liver and kidney. The active form of HGFA was detected in the kidney but not in the liver, lung, and spleen (Fig. 3, lane 3). These results indicate that the active form of HGFA is generated exclusively in the injured tissues.

Heparin Binding Properties of the Precursor and Active Forms of Rat HGFA—Binding to heparin or to heparan sulfate is postulated to modulate the localization of several growth factors, including HGF (29, 30). Thus we assessed the heparin binding properties of HGFA. Rat plasma or serum was applied

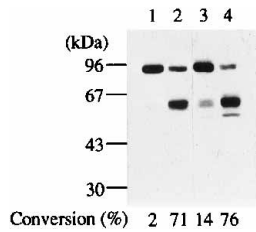


FIG. 2. **Abrogation of HGF-converting activity in the injured liver by an anti-HGFA antibody.** A single-chain HGF was incubated with homogenate of injured rat liver and analyzed by immunoblotting using the P-1 mAb that was raised against the heavy chain of HGF. Lane 1, no incubation; lane 2, 37 °C for 8 h; lane 3, 37 °C for 8 h, pretreated with anti-HGFA, P1-4; lane 4, 37 °C for 8 h, pretreated with P-5, which does not react with HGFA (as a negative control).

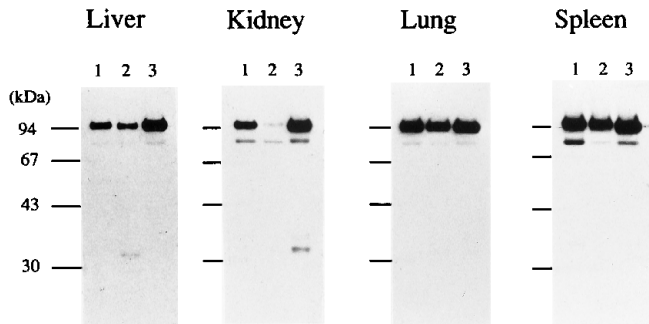


FIG. 3. **Tissue-derived HGFA after hepatic or renal injury.** Soluble proteins (40 mg) of tissue homogenates from liver, kidney, lung, and spleen were fractionated on P1-4 mAb affinity column and analyzed by immunoblotting. Lane 1, normal; lane 2, 12 h after CCl_4 treatment; lane 3, 12 h after HgCl_2 treatment. The band of 82 kDa probably corresponds to a degradation product of the precursor (27).

to a heparin-Sepharose column; the bound proteins were eluted in a stepwise manner with increasing concentrations of NaCl and then analyzed by immunoblotting (Fig. 4). When we analyzed rat plasma (Fig. 4A), a band appeared at the position corresponding to 96 kDa. Thus, rat plasma contained exclusively the inactive precursor of HGFA. The precursor was eluted at the NaCl concentration of 100–200 mM, indicating that it interacts with heparin only weakly near the physiological salt concentration. On the analysis of rat serum (Fig. 4B), a band appeared at the 33-kDa position, indicating that the rat serum contained mostly the active form of HGFA. It is noteworthy that the active form was eluted at the NaCl concentration of 300–600 mM. These results indicate that, after it was activated, HGFA acquired a heparin binding capacity near the physiological salt concentration.

DISCUSSION

The biological activities of HGF have been shown to be localized to injured tissues by proteolytic processing *in vivo* (20). In that study, when the rat was treated with hepatotoxin, HGF was converted to its active form in the liver but not in the kidney, lung, or spleen. Similarly, when the rat was treated with nephrotoxin, HGF was activated in the kidney but not in the liver, lung, or spleen. We found that HGF-converting activity was induced in the injured liver (20). Since it appears likely that this activity plays a key role in regulation of HGF activities in the injured tissues, it is important to identify the protease responsible for this activity.

In the present study, we clearly demonstrated that the HGF-converting activity in the injured liver was abrogated by treatment with HGFA-specific antibody. In addition, we found that the active form of HGFA was generated in the liver and kidney after induction of hepatic and renal injury, respectively. This generation was not observed in the uninjured tissues. These

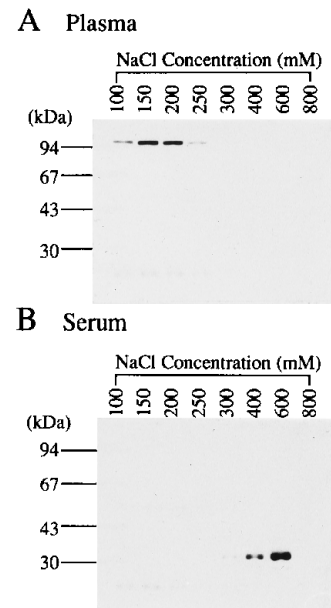


FIG. 4. **Heparin binding properties of the precursor and active forms of rat HGFA.** Rat plasma (A) or serum (B) was diluted and applied to a heparin-Sepharose column. After washing was carried out, the bound proteins were eluted in a stepwise manner with buffer containing increasing concentrations of NaCl (100–800 mM) and analyzed by immunoblotting.

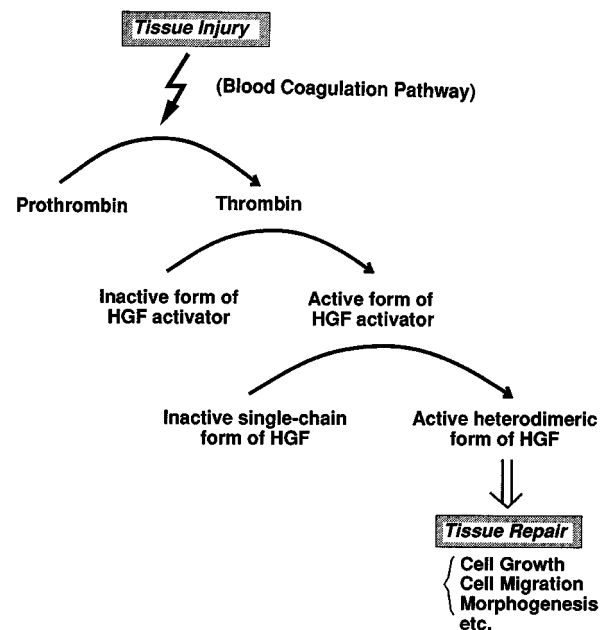


FIG. 5. **Proteolytic cascade for activation of HGF in response to tissue injury.**

results indicate that HGFA is most likely the key enzyme that is involved in the locally restricted generation of active HGF in the injured tissues.

The properties of HGFA well explain the presence of HGFA activity in the injured tissues and its absence in normal tissues. HGFA was first identified in bovine serum (21) and human serum (22). The cDNA cloning revealed that its sequence was homologous (39%) to that of blood coagulation factor XII (22). HGFA is a plasma protein produced in the liver. It circulates in the blood as an inactive zymogen that is converted into the active form by thrombin during blood coagulation (27). Because tissue injury often leads to the activation of the blood coagulation pathway (31), the zymogen form of HGFA is converted into

the active enzymes to activate HGF in injured tissues. To retain the activity of HGFA in the close vicinity of the injured locus, it would be favorable if HGFA protein were not freely diffusible. The active form of HGFA appears to be associated with the cell surface. In our previous study, we detected HGFA activity in a primary culture of hepatocytes under serum-free conditions (15). The activity was cell-bound, because the single-chain HGF was not converted to the heterodimer during incubation with the serum-free conditioned medium of the culture (32). In the present study, we found that HGFA acquired a heparin binding ability after it was activated. These results indicate that the zymogen of HGFA is rather diffusible, whereas the activated form can associate with cell surface heparin-like molecules and is prevented from free diffusion. This property of HGFA probably ensures its localized action on HGF. The binding of HGFA with heparin-like molecules has another merit in activating HGF, because HGF is also associated with heparin-like molecules on the cell surface (16, 29).

In conclusion, a proteolytic cascade plays a key role in the activation of HGF in response to tissue injury (Fig. 5). Recently, Uehara *et al.* (33) and Schmidt *et al.* (34) reported that disruption of the HGF gene in mice caused embryonic lethality, indicating that HGF is an essential factor for mouse development. During development, the activity of growth/differentiation factors must be spatially restricted. Thus, a proteolytic activation system for HGF appears to be required also during the development. Further study is necessary to determine whether or not HGFA also functions as a HGF-converting enzyme during mammalian development.

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