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

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Hepatocyte growth factor (HGF), 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 this enzyme at the site of tissue injury.

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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 monodisperse antibody was added at 400 μg/ml. After gentle agitation at 4 °C for 24 h, 2 μg of single-chain HF 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) 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. HF 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-HF 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. HF serum was prepared from the same animal and treated with 1 mM PMSF. NaCl concentrations were adjusted to 0.65 M HF. 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-HF monoclonal antibody A-1.

RESULTS

Purification and Characterization of Rat HF Activator—Rat HF was purified from rat serum by the same procedure as that described for the purification of bovine HF. 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 was obtained from 100 ml of rat serum. The purified protein converted recombinant human single-chain HF at low doses, comparable with human HF (Fig. 1A). The sensitivity of rat HF to various serum protease inhibitors was the same as that of bovine and human HF (data not shown).

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

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

Presence of the Active Form of HF in Injured Tissues—HF 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 formation of HF in injured and uninjured tissues after induction of hepatic or renal injury (Fig. 3). The amount of HF 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 HF 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 HF increased in the liver and kidney. The active form of HF 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 HF is generated exclusively in the injured tissues.

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