

Existence of two nonlinear elimination mechanisms for hepatocyte growth factor in rats

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Liu, Ke-Xin, Yukio Kato, Motohiro Kato, Tai-Ichi Kaku, Toshikazu Nakamura, and Yuichi Sugiyama. Existence of two nonlinear elimination mechanisms for hepatocyte growth factor in rats. *Am. J. Physiol.* 273 (*Endocrinol. Metab.* 36): E891–E897, 1997.—Nonlinearity in the overall elimination of hepatocyte growth factor (HGF) was examined in rats. After intravenous administration, the plasma clearance (CL_{plasma}) of HGF exhibited a dose-dependent biphasic reduction with high- and low-affinity components. If we consider our previous finding that both receptor-mediated endocytosis (RME) and a low-affinity uptake mechanism, probably mediated by heparan sulfate proteoglycan (HSPG), in the liver are major HGF clearance mechanisms, it may be that saturation of CL_{plasma} at lower and higher doses represents saturation of RME and HSPG-mediated uptake, respectively. At an HGF dose (1.46 nmol/kg), which completely saturates the high-affinity component, CL_{plasma} was almost completely reduced when HGF was premixed with heparin. However, CL_{plasma} was reduced by heparin to, at most, one-fifth that after HGF alone in a dose near the linear range (3.66 pmol/kg). Saturation of CL_{plasma} for HGF premixed with heparin was monophasic and nonlinear only at the lowest HGF doses. In vitro, high-affinity binding of [³⁵S]heparin to HGF was found, showing that one HGF molecule binds to the penta- or hexasaccharide unit. Because mitogenic activity of HGF has been reported in the presence of heparin, these results suggest that heparin mainly inhibits low-affinity HGF uptake by complexing with HGF, whereas its effect on RME is relatively minor.

receptor-mediated endocytosis; heparin

HEPATOCYTE GROWTH FACTOR (HGF) was first identified as a potent mitogen for mature hepatocytes and is now recognized as a mitogen for a variety of types of epithelial cells (18). The biological effect of HGF is not restricted to its mitogenic activity, but HGF is identical to the scatter factor that acts as a motogen stimulating the migration of epithelial cells (31). HGF is also a morphogen for epithelial cells, and it induces a multicellular architecture (20) as well as being a potent angiogenic factor capable of inducing endothelial cells to proliferate and migrate (3). The biological activity of HGF is exerted through its binding to a specific receptor. The HGF receptor is a protooncogene *c-met* product (2, 6) and is expressed on ubiquitous epithelial cells (25, 29). It has been suggested that HGF binding to the receptor induces receptor dimerization, resulting in reciprocal *trans*-phosphorylation of each receptor and subsequent interaction with the other cytoplasmic effectors (1, 11). HGF has an affinity for heparin and can bind to the so-called heparin-like substance on the cell

surface and/or extracellular matrix (17, 32). Lyon et al. (16) demonstrated that the heparan sulfate proteoglycan (HSPG) derived from the liver binds to HGF. Thus HSPG is thought to be responsible for the binding to HGF as a heparin-like substance. Both a heparin-binding domain and a receptor-binding domain on the HGF molecule have been identified at the NH₂-terminal half of the α -chain. The former is located on the NH₂-terminal hairpin loop and the second Kringle domain, whereas the latter is within the region of the hairpin loop and the first Kringle domain (19).

HGF also exhibits biological activity in vivo in several types of animal with experimentally induced liver and kidney disease (9, 27). However, the dose of HGF needed to produce a pharmacological effect is usually high (>1.22 nmol/kg) except when HGF is administered through the portal vein (4, 10), in which 0.61–3.05 pmol/kg HGF stimulates hepatocyte growth. One of the reasons that such a high dose is needed may be the short plasma half-life of HGF (~4 min) (12). For the clinical application of HGF, it is important to clarify the mechanism of its elimination from the circulating plasma. We have been studying the clearance mechanism of HGF and suggested that both receptor-mediated endocytosis (RME) and a low-affinity uptake mechanism, probably mediated by a cell-surface HSPG, mainly contribute to the systemic clearance of HGF under tracer conditions (12–14). However, its nonlinear pharmacokinetic behavior has not been completely clarified, and so in the present study, we investigated a number of dose-dependent pharmacokinetic profiles.

To display the pharmacological activity of HGF in vivo, it is important to develop an efficient drug-delivery system for HGF (8, 27). Because the HSPG on the cell surface may mediate the uptake of HGF, its plasma clearance can be reduced when HGF is premixed with heparin to form a heparin-HGF complex (8). A mitogenic response by hepatocytes to HGF can be observed in the presence of heparin (8, 32, 33), suggesting that HGF can bind to its receptor even when it forms a complex with heparin. We previously reported the reduction in HGF clearance after its coinjection with heparin in rats (8). In that study, however, because trichloroacetic acid precipitation was used to determine the plasma concentration of HGF, the experiment was carried out within a short time (<30 min) of its administration (8). In this study, we used enzyme immunoassay (EIA) to investigate the plasma concentration-time profile of HGF for a long period (~48 h) to examine the effect of heparin on HGF disposition.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 250 g (Nisseizai, Tokyo, Japan) were used. All animals received humane care in compliance with the National Research Council's criteria for humane care as outlined in "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication no. 86-23, revised 1985).

Materials. Porcine intestinal mucosa heparin with a molecular mass of 18–23 kDa (185.5 U/mg) was purchased from Sigma (St. Louis, MO); human recombinant HGF was purified from a culture medium of C-127 cells transfected with plasmid containing human HGF cDNA (23).

Pharmacokinetic analysis of HGF or heparin-HGF complex. Heparin dissolved in saline was incubated with HGF for 50 min at 25°C (8). With the animals under light ether anesthesia, HGF alone or the heparin-HGF mixture was administered through the penile vein, and at specified times, blood samples were withdrawn through the jugular vein without cannulation. Plasma HGF concentrations were determined using an EIA kit (Institute of Immunology, Tochigi, Japan).

The plasma concentration (C_p)-time profiles of HGF after intravenous administration were fitted to the following two-exponential equation using a nonlinear iterative least-squares method (12)

$$C_p = A \exp(-\alpha t) + B \exp(-\beta t) \quad (1)$$

where α and β are the apparent rate constants. A and B are the corresponding zero-time intercepts, and t is time. The input data were weighted as the reciprocal of the square of the observed values, and the algorithm used for fitting was the damping Gauss Newton method.

The area under the plasma concentration-time curve from time 0 to t [$AUC_{(0-t)}$] was calculated as

$$\begin{aligned} AUC_{(0-t)} &= \int_0^t C_p dt \\ &= A/[1 - \exp(-\alpha t)] + B/[1 - \exp(-\beta t)] \end{aligned} \quad (2)$$

The area under the plasma concentration-time curve from time 0 to infinity [$AUC_{(0-\infty)}$] was calculated as

$$AUC_{(0-\infty)} = A/\alpha + B/\beta \quad (3)$$

The plasma clearance (CL_{plasma}) was calculated from

$$CL_{\text{plasma}} = \text{dose}/AUC_{(0-\infty)} \quad (4)$$

Ligation of portal vein and hepatic artery. With the animals under light ether anesthesia, both the portal vein and hepatic

artery were ligated before intravenous injection of HGF. In a sham operation, rats were anesthetized, and laparotomies were performed without ligation.

In vitro binding of [^{35}S]heparin to HGF. The protamine-affinity fraction of [^{35}S]heparin (15–25 mCi/g, Amersham, Arlington Heights, IL) was chromatographed on a Sephadex G-100 column (114 cm \times 1.5 cm ID) at a flow rate of 0.18 ml/min using phosphate-buffered saline as elution buffer (30). Blue dextran (Pharmacia, Uppsala, Sweden), fluorescein-dextran with molecular masses of 40 and 10 kDa (Cosmo-Bio, Tokyo, Japan), [^{14}C]inulin (New England Nuclear, Boston, MA), and $^3\text{H}_2\text{O}$ (New England Nuclear) were also separately eluted as molecular mass markers. The volume of each fraction was 2.0 ml. Fractions 44–46 and 56–58 were collected as [^{35}S]heparin with molecular masses of 21–23 and 12–13 kDa, respectively. The molecular mass of the [^{35}S]heparin was determined from a plot of k_{avg} vs. the logarithm of the molecular mass of each marker where

$$k_{\text{avg}} = (V_e - V_o)/(V_t - V_o) \quad (5)$$

The V_e , V_o , and V_t were peak fraction numbers for each molecular mass marker, Blue dextran, and $^3\text{H}_2\text{O}$, respectively. [^{35}S]heparin dissolved in the buffer containing 120 mM NaCl, 4.8 mM KCl, 1.0 mM KH_2PO_4 , 1.2 mM MgSO_4 , 5.0 mM glucose, 2.2 mM CaCl_2 , and 20 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 7.4) was incubated with HGF (50 nM) for 50 min at 25°C. Total and unbound radioactivity was determined by ultrafiltration with a molecular mass limitation of 30 kDa. The binding parameters were obtained by fitting the data to the following equation using an iterative nonlinear least-squares method

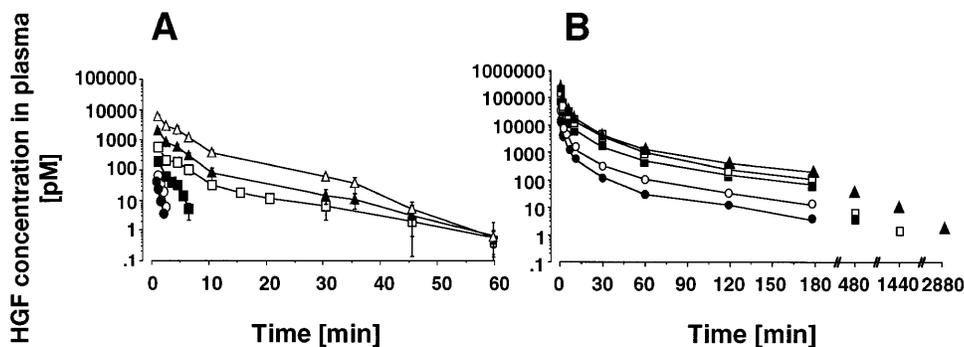
$$C_b = n P_t C_f / (K_d + C_p) + \alpha P_t C_f \quad (6)$$

where C_b , C_f , n , P_t , K_d , and α are the concentration of heparin bound to HGF, unbound heparin concentration, specific binding capacity, HGF concentration, dissociation constant, and proportional constant for nonspecific binding, respectively.

RESULTS

Nonlinear elimination of HGF. After administration of various doses of HGF (1.22 pmol/kg–12.2 nmol/kg) as bolus intravenous injections, plasma concentration-time profiles were investigated using EIA (Fig. 1). At HGF doses of <12.2 pmol/kg, HGF in plasma rapidly disappeared, whereas at >12.2 pmol/kg, such plasma disappearance was delayed (Fig. 1). From the data shown in Fig. 1, CL_{plasma} was obtained (Fig. 2). CL_{plasma} exhibited a dose-dependent reduction with increasing dose (Fig. 2). This reduction was biphasic, showing both

Fig. 1. Plasma concentration-time profiles of hepatocyte growth factor (HGF) after intravenous administration of various doses of HGF. After intravenous administration of 1.22 (●), 3.66 (○), 12.2 (■), 36.6 (□), 122 (▲), or 366 (△) pmol/kg HGF (A) and 1.46 (●), 3.66 (○), 6.10 (■), 8.66 (□), or 12.2 (▲) nmol/kg HGF (B) as a bolus dose, plasma concentration-time profiles were determined using enzyme immunoassay (EIA). Each point and vertical bar represent mean \pm SE of 3 rats. Plasma clearance (CL_{plasma}) of HGF was calculated based on these time profiles and is shown in Fig. 2.



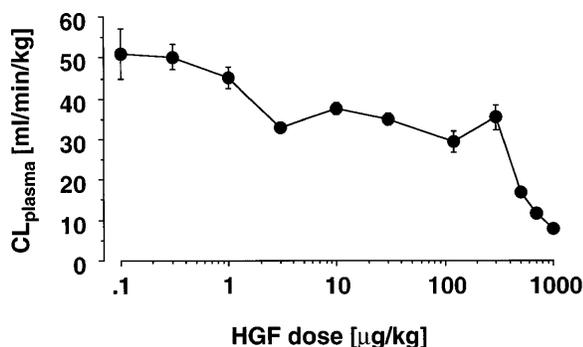


Fig. 2. Two saturable components in CL_{plasma} of HGF. From plasma concentration-time profiles of HGF shown in Fig. 1, CL_{plasma} of HGF in normal rats was estimated and plotted against dose. Each point and vertical bar represent mean \pm SE of 3 rats. Vertical bar is not shown when SE value is smaller than symbol.

a high-affinity component saturated at relatively low doses (1–3 $\mu\text{g}/\text{kg}$, 12.2–36.6 pmol/kg) and a low-affinity component saturated at relatively higher doses (300–1,000 $\mu\text{g}/\text{kg}$, 3.66–12.2 nmol/kg) (Fig. 2).

Elimination profile of HGF in rats with ligated portal vein and hepatic artery. To directly demonstrate the importance of the liver for the plasma clearance of HGF, the elimination profile of HGF in plasma was examined in rats after ligation of both the portal vein and hepatic artery (Fig. 3). The plasma disappearance of HGF at all doses examined was delayed in ligated rats compared with the control (sham-operated) rats (Fig. 3). CL_{plasma} in ligated rats was 14.5 ± 1.1 , 9.18 ± 0.90 , 0.903 ± 0.349 , and $0.538 \pm 0.362 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ (means \pm SE, $N = 3$) at HGF doses of 1.22, 3.66, and

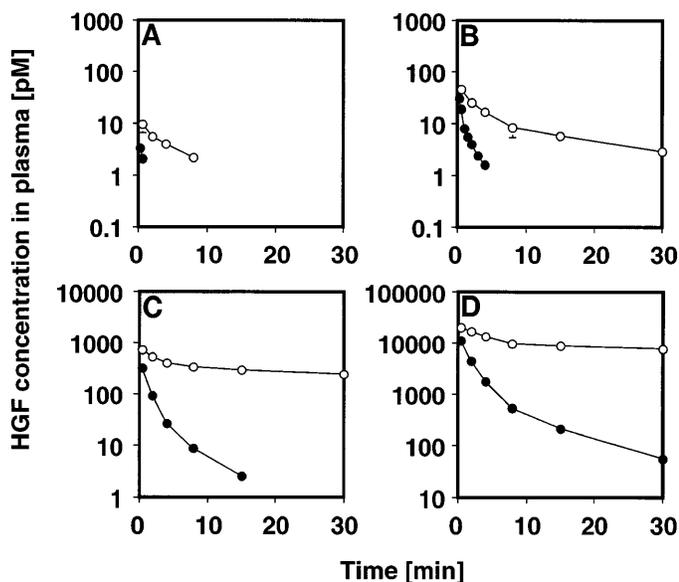


Fig. 3. Elimination profile of HGF in rats with ligated portal vein and hepatic artery. With animals under light ether anesthesia, both portal vein and hepatic artery were ligated (○), followed by intravenous administration of bolus doses of HGF: 1.22 (A), 3.66 (B), or 36.6 pmol/kg (C) or 1.10 nmol/kg (D). Plasma concentration-time profiles were determined using EIA. As a control experiment, plasma concentration-time profile of HGF was also examined in sham-operated rats (●). Each point and vertical bar represent mean \pm SE of 3 rats.

36.6 pmol/kg and 1.10 nmol/kg, respectively, also showing a dose-dependent reduction.

Effect of heparin on nonlinear elimination of HGF. HGF was first mixed with heparin (0–20 mg/kg) and then given intravenously (Fig. 4A) at an HGF dose of 1.46 nmol/kg (120 $\mu\text{g}/\text{kg}$) when the high-affinity component of CL_{plasma} was almost completely saturated (Fig. 2). Plasma concentration-time profile of HGF after injection of a mixture with heparin at 0.004 mg/kg was almost identical to that after injection of HGF alone (Fig. 4). At >0.02 mg/kg heparin, the plasma disappearance of HGF was delayed after injection of heparin-HGF mixture (Fig. 4) compared with that after injection of HGF alone. This effect was heparin dose dependent (Fig. 4) and reached a maximum when the dose of heparin was 0.4 mg/kg (Fig. 4). From the data shown in Fig. 4A, a kinetic parameter, the $AUC_{(0-180)}$, representing the exposure of HGF in plasma was determined (Fig. 4B). The $AUC_{(0-180)}$ was $0.0423 \pm 0.0039 \text{ nmol} \cdot \text{min} \cdot \text{ml}^{-1}$ after injection of HGF (1.46 nmol/kg) alone and increased ~ 21 -fold ($0.899 \pm 0.079 \text{ nmol} \cdot \text{min} \cdot \text{ml}^{-1}$) after injection of a mixture with 0.4 mg/kg heparin (Fig. 4B).

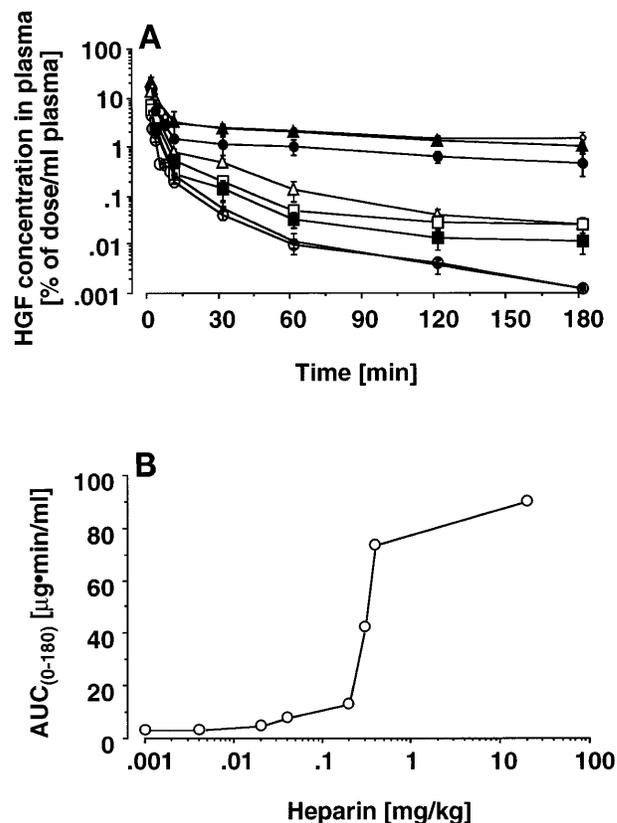
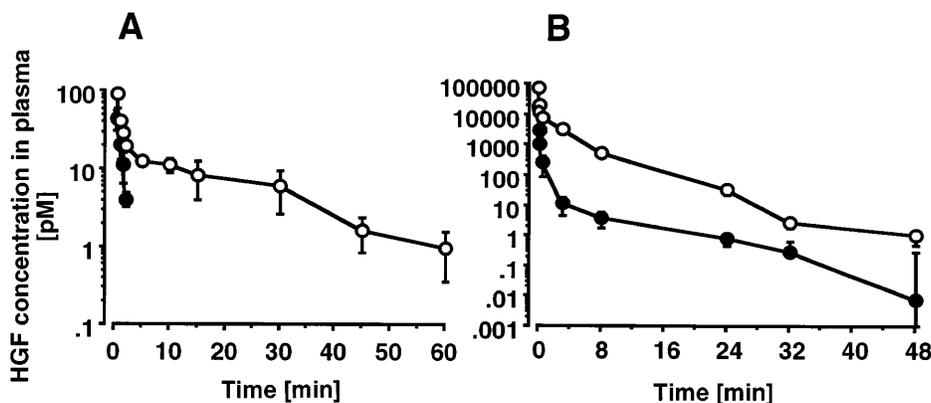


Fig. 4. Plasma concentration-time profiles (A) and area under curve [$AUC_{(0-180)}$; B] of HGF after intravenous administration of HGF alone or HGF premixed with heparin. A: HGF was mixed with heparin to give final heparin doses of 0 (large ○), 0.004 (small ■), 0.02 (large ■), 0.04 (□), 0.2 (△), 0.3 (●), 0.4 (▲), or 20 (small ○) mg/kg and then injected intravenously. Plasma concentration-time profiles of HGF were determined using EIA and normalized for injected dose. B: from data shown in A, $AUC_{(0-180)}$ was calculated using Eq. 2. Each point and vertical bar represent mean \pm SE of 3 rats.

Fig. 5. Plasma concentration-time profiles of HGF after intravenous administration of HGF alone or HGF premixed with heparin. At HGF doses of 3.66 pmol/kg (A) and 1.46 nmol/kg (B), HGF alone (●) or HGF premixed with sufficient heparin (0.4 mg/kg, ○) was injected intravenously. Plasma concentration-time profiles of HGF were determined by EIA. Each point and vertical bar represent mean \pm SE of 3 rats. Pharmacokinetic parameters obtained are shown in Table 1.



The plasma concentration-time profiles of HGF after intravenous injection of HGF alone or a heparin-HGF mixture were determined for a longer period (~ 48 h) to accurately estimate CL_{plasma} (Fig. 5). At an HGF dose of 3.66 pmol/kg (0.3 $\mu\text{g}/\text{kg}$) near the linear dose range for CL_{plasma} (Fig. 2), HGF was first mixed with enough heparin (0.4 mg/kg), and the mixture was then given intravenously (Fig. 5A). The plasma disappearance of HGF was also delayed compared with that after an injection of 3.66 pmol/kg HGF alone (Fig. 5A). However, the reduction in CL_{plasma} at an HGF dose of 3.66 pmol/kg was not so marked (Fig. 5A, Table 1) compared with the HGF dose of 1.46 nmol/kg (Fig. 5B, Table 1). At an HGF dose of 3.66 pmol/kg, CL_{plasma} after administration of HGF with heparin was 21% that after administration of HGF alone (Table 1). On the other hand, at an HGF dose of 1.46 nmol/kg, CL_{plasma} of HGF with hepa-

rin was only 2.4% that after administration of HGF alone (Table 1). The saturable component of CL_{plasma} was estimated by subtracting CL_{plasma} at 1.46 nmol/kg from that at 3.66 pmol/kg for both HGF alone and the heparin-HGF mixture; this was $\sim 10 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ for the heparin-HGF mixture, approximately one-half that for HGF alone (Table 1).

Binding of [^{35}S]heparin and HGF. The binding of the protamine-affinity fraction of [^{35}S]heparin and HGF was determined by ultrafiltration (Fig. 6). For both [^{35}S]heparins with molecular masses of 21–23 and 12–13 kDa, saturable and nonsaturable components could be observed for the binding with HGF (Fig. 6). K_d values were 0.310 ± 0.130 and 0.487 ± 0.292 nM, n values were 0.0862 ± 0.0126 and 0.121 ± 0.024 mol of heparin/mol of HGF, and α values were 0.0115 ± 0.0012 and 0.0165 ± 0.0018 (means \pm calculated SD) for the 21- to 23- and 12- to 13-kDa [^{35}S]heparins, respectively (Fig. 6).

Table 1. Nonlinear pharmacokinetic parameters of HGF and HGF premixed with heparin

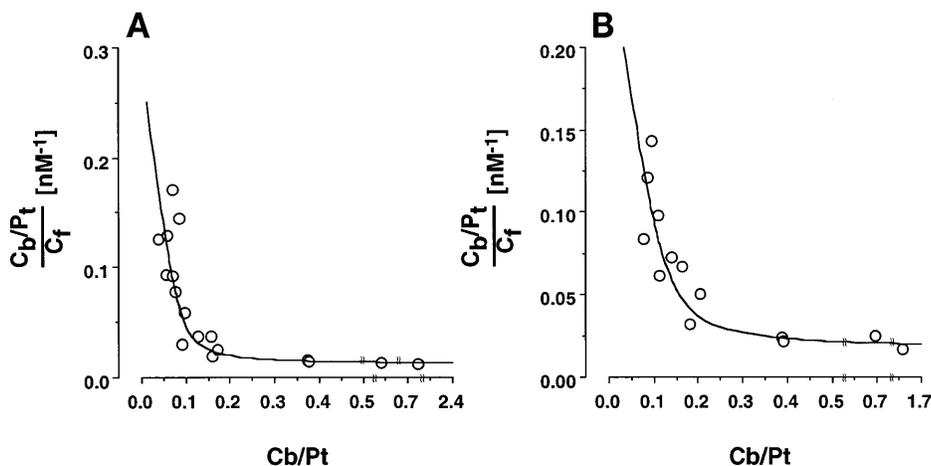
Dose of HGF	Dose of Heparin, mg/kg	CL_{plasma} , $\text{ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$
3.66 pmol/kg	0	50.2 ± 3.15
1.46 nmol/kg	0	29.4 ± 2.56
Saturable component		19.3 ± 6.01
3.66 pmol/kg	0.4	10.5 ± 3.83
1.46 nmol/kg	0.4	0.691 ± 0.245
Saturable component		9.85 ± 3.65

Values are means \pm SE. HGF, hepatocyte growth factor; CL_{plasma} , plasma clearance.

DISCUSSION

We have investigated the elimination mechanism of HGF and come to several conclusions. The major clearance organ for HGF is the liver (12), and the clearance mechanism for HGF consists of at least two systems, RME and a low-affinity uptake mechanism probably through a cell-surface HSPG (13, 14). Because the nonlinear elimination profile of HGF from the circulation has never been reported previously, we

Fig. 6. Scatchard plot representing binding of [^{35}S]heparin to HGF. Protamine-affinity fraction of [^{35}S]heparin with a molecular weight of 21–23 (A) or 12–13 kDa (B) was incubated with HGF at 25°C for 50 min, and [^{35}S]heparin binding to HGF was determined by ultrafiltration. Lines in figure represent calculated curves obtained by fitting to Eq. 6. C_b , heparin bound to HGF; C_f , unbound heparin concentration; P_t , HGF concentration.



analyzed the plasma concentration-time profiles of HGF after intravenous administration of several different doses (Figs. 1 and 3). The present study supports the liver as the major clearance organ for HGF at any of the doses of HGF examined, since the disappearance of plasma HGF was significantly delayed in rats with their portal vein and hepatic artery ligated (Fig. 3).

When we consider that RME contributes to HGF clearance (12–14), it may be that its plasma elimination exhibits nonlinearity because of saturated receptor binding and/or subsequent endocytosis. Actually, CL_{plasma} showed biphasic saturation with increasing HGF doses (Fig. 2); e.g., CL_{plasma} was reduced at 12.2–36.6 pmol/kg and 3.66–12.2 nmol/kg (Fig. 2). This result suggests that the clearance mechanism consists of at least two systems, a high-affinity clearance site and a low-affinity one. The saturation in CL_{plasma} was observed at 12.2–36.6 pmol/kg, at which the plasma concentration ranged from 10 to 100 pM (Fig. 1). Because the equilibrium dissociation constant of the HGF receptor is 20–40 pM (7), this result suggests that the saturation at the lower dose range (12.2–36.6 pmol/kg) comes from saturation of RME. On the other hand, CL_{plasma} also exhibited saturation over the dose range 3.66–12.2 nmol/kg (Fig. 2). This can be explained if we consider that not only RME but also the low-affinity uptake mechanism, probably mediated by HSPG, can be saturated at this higher HGF dose range.

If the nonlinearity in CL_{plasma} observed at the lower dose range (12.2–36.6 pmol/kg) results from the saturation of RME, the HGF clearance at the much higher dose range should be almost exclusively governed by the low-affinity uptake mechanism and not RME. Under such conditions, CL_{plasma} should be almost completely stopped when HGF prebound to heparin is injected, since the heparin-binding site on the HGF molecule is occupied by the heparin so that the heparin-HGF complex cannot bind to the HSPG (8). HGF has an affinity for heparin (18, 22, 24). In the present study, we showed that HGF can bind to heparin with high affinity and exhibits an equilibrium dissociation constant of 0.3–0.5 nM (Fig. 6). When we gave intravenous HGF (1.46 nmol/kg) prebound to sufficient heparin (0.4 mg/kg), CL_{plasma} was almost completely reduced to zero compared with that after the injection of HGF alone (Fig. 5B, Table 1). This result also suggests that the low-affinity clearance site, which cannot be saturated at the lower dose range (~3.66 nmol/kg), represents this HSPG. The details of the mechanism of this low-affinity clearance site are still unknown. In an earlier study, we found that part of the ^{125}I -HGF internalization is not saturated even in the presence of unlabeled HGF (135 pM) and is also insensitive to phenylarsine oxide, an inhibitor of RME, in perfused rat liver (12). Thus the sensitivity of each clearance site to the RME inhibitor may be different. However, we cannot deny that the low-affinity component is also mediated by an RME-like mechanism and further studies are needed to clarify the mechanism of the low-affinity component.

On the other hand, when HGF near the linear dose range (3.66 pmol/kg) was premixed with heparin and administered intravenously, CL_{plasma} was not completely reduced but was ~20% of that after administration of HGF (3.66 pmol/kg) alone (Fig. 5A, Table 1). This can be explained by considering that HGF prebound to heparin can still bind to the HGF receptor and be eliminated through RME. Actually, even when HGF was premixed with a sufficient amount of heparin, a saturable component in CL_{plasma} of HGF could still be observed (Table 1). In addition, we and others (8, 32, 33) have reported that the mitogenic response to HGF can be observed even in the presence of heparin in primary cultured rat hepatocytes. These results support our hypothesis that HGF bound to heparin can still bind to its receptor. However, the saturable component in CL_{plasma} after administration of heparin-HGF complex was at most one-half that after administration of HGF alone (Table 1). When we consider that this saturable portion mainly reflects RME, this result suggests that the efficiency in RME of HGF prebound to heparin is approximately one-half that of HGF alone. On the other hand, the half-effective concentration of the mitogenic effect of HGF in cultured rat hepatocytes increased two- to threefold after the addition of heparin (8), suggesting that the affinity of heparin-HGF complex for the HGF receptor is also one-half that of HGF alone. Thus it can be speculated that the relatively lower affinity of heparin-HGF complex for the receptor results in the lower saturable component in CL_{plasma} . Actually, Naka et al. (21) analyzed the interaction between HGF and its receptor in the presence of heparin and found that heparin added during the binding of ^{125}I -HGF to its receptor significantly reduced the cross-linking of ^{125}I -HGF to the HGF receptor. This result implies that heparin can inhibit the receptor binding of HGF. Nevertheless, they also showed in their study that the mitogenic response to HGF was exhibited in the presence of heparin. Thus, although it is likely that the affinity of heparin-HGF complex for the receptor is relatively low, this complex still exhibits the biological activity of HGF.

It has been reported that HGF exhibits marked pharmacological activity in several types of experimental animal models of liver and kidney dysfunction (4, 9, 10, 27). However, a large dose (>1.22 nmol/kg) is needed to obtain any effect in vivo (9, 27), although biological activity can be observed at very low (~100 pM) concentrations in vitro (7, 32). Therefore, for its clinical application, we need to develop a drug-delivery system so that the pharmacological effects can be obtained at much lower doses. We suggest that the heparin-HGF complex may be a candidate for such a drug-delivery system (8). In this study, CL_{plasma} of HGF can be reduced to 2% of that of the controls by complex formation with heparin at an HGF dose (1.46 nmol/kg) within the range where its pharmacological activity can be observed (Table 1). This effect of heparin on HGF clearance is heparin dose dependent and reaches a maximum at a heparin dose of 0.4 mg/kg (Fig. 3), corresponding to 74 U/kg. Because an intravenous

clinical dose of heparin is usually 100 U/kg (5), this dose of heparin is within the clinical dose range. Our present study therefore suggests that clinical doses of heparin can almost completely block HGF clearance.

CL_{plasma} also showed nonlinearity in rats with ligated portal vein and hepatic artery (Fig. 3). This saturation in CL_{plasma} was almost complete and observed at a dose of $<1.22 \text{ nmol/kg}$ ($15 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ at 1.22 pmol/kg and $0.5 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ at 1.10 nmol/kg). This result suggests that the clearance mechanism in extrahepatic organs mainly consists of the high-affinity component mediated by RME. Expression of the HGF receptor is not restricted in the liver but is also observed in extrahepatic organs such as kidney, spleen, and lung (25, 29). Therefore, clearance via these organs may be part of the extrahepatic organ clearance. However, we should be careful about the absolute value of CL_{plasma} under such unphysiological conditions. In order to estimate more accurately the contribution of the liver, a further study needs to be done.

Our [^{35}S]heparin binding study with HGF revealed that the binding capacity of HGF (n) was 0.086 mol heparin/mol HGF for 21- to 23-kDa [^{35}S]heparin (Fig. 6). This means that 1 heparin molecule can bind to ~ 12 HGF molecules, and 1 unit in the heparin molecule, which can bind to 1 HGF molecule, should have a molecular weight of 1.9 kDa (corresponding to a hexasaccharide). On the other hand, n was 0.12 mol heparin/mol HGF for 12- to 13-kDa [^{35}S]heparin (Fig. 6), indicating that one unit in the heparin molecule binding to one HGF molecule should have a molecular weight of 1.5 kDa (corresponding to a pentasaccharide). Lyon et al. (16) demonstrated that the minimal size binding component in heparan sulfate derived from the liver is a hexasaccharide. Heparan sulfate derived from liver possesses more heparin-like properties than a heparan sulfate species derived from other sources (15). Zioncheck et al. (33) reported that low concentrations of sulfated oligosaccharides of sufficient length (6 glucose units) induce dimerization of HGF and also increase its mitogenic effect on cultured rat hepatocytes. Both our findings and their report can be understood if a penta- or hexasaccharide can bind to an HGF molecule and stimulate its conformational change, resulting in dimerization of HGF. It is well known that receptor binding and the subsequent mitogenic effect of fibroblast growth factor (FGF) are regulated by HSPG on the cell surface (26, 28). One of the proposed mechanisms for such regulation by HSPG is that binding of FGF with heparan sulfate may induce a conformational change in FGF, resulting in its receptor binding (26, 28). Such a regulation may also occur in the interaction between HGF and its receptor.

In conclusion, the plasma clearance of HGF is governed by two mechanisms, RME and a low-affinity uptake mechanism, probably mediated by a cell-surface HSPG, and exhibits nonlinearity that stems from the saturation of these mechanisms as the dose increases. HGF premixed with a clinical dose of heparin shows a much lower plasma clearance compared

with HGF alone, mainly by preventing binding to HSPG.

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