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Running title:

Pharmacokinetic model of HGF after single and repeated doses

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Document statistics:

Number of text pages: 41

Number of tables: 2

Number of figures: 4

Number of references: 46

Number of words:

Abstract: 200

Introduction: 611

Discussion: 2037

Additional Supporting Information may be found in the online version of this article.

Abbreviations: HGF, hepatocyte growth factor; FITC, fluorescein-4-isocyanate; PBS, phosphate-buffered saline; RME, receptor-mediated endocytosis; ARF, acute renal failure; BUN, blood urea nitrogen; ALT, alanine transaminase

Submitted to: Journal of Pharmaceutical Sciences

ABSTRACT

Hepatocyte growth factor (HGF) is under development for treatment of renal failure. This study was designed to clarify changes in HGF pharmacokinetics in renal failure and to establish a pharmacokinetic model applicable to single and repeated doses. The plasma concentration profile in mice with glycerol-induced acute renal failure was similar to that in normal mice, indicating a minimal contribution of kidney to systemic clearance of HGF. Nevertheless, accumulation of FITC-labeled HGF in renal tubules in both cases suggests the occurrence of efficient endocytosis of HGF in kidney. A pharmacokinetic model including plasma and liver compartments was constructed, incorporating both high- and low-affinity receptors for association and subsequent endocytosis of HGF, because HGF is eliminated via specific receptor c-Met and heparin-like substance. The model well explained the plasma concentration profiles at all doses examined after bolus injection in animals and humans, and those during infusion in rodents. It includes externalization of receptors, which is negatively regulated by HGF, and can explain the gradual increase in trough concentration during repeated dosing in monkeys. Overall pharmacokinetic profiles of HGF are governed by at least two receptors and are well described by this pharmacokinetic model, which should assist in safe management of clinical trials.

Keywords: Pharmacokinetics/pharmacodynamics, Proteins, Receptors, Hepatic clearance, Elimination, Hepatocytes, Distribution

INTRODUCTION

Hepatocyte growth factor (HGF) was first identified as a potent mitogen for matured hepatocytes and is now recognized as a pleiotropic factor with a range of biological activities in epithelial and endothelial cells.[1,2] Systemic administration of HGF has various beneficial effects in renal failure models in experimental animals. In a mouse model of obstructive nephropathy, for example, HGF attenuates the progression of interstitial fibrosis with a concomitant reduction in tubular apoptosis and an increase in tubular proliferation.[3-5] In nephritic mice, HGF protects podocytes from nephron loss, leading to a reduction of proteinuria.[6,7] In rats, HGF gene transfer reduces cyclosporin A-induced tubulointerstitial injury.[8] Due to such promising biological activities and the limited availability of therapeutic agents for renal failure, there is great current interest in HGF as a potential treatment for renal failure.

HGF has also been reported to prevent fulminant hepatic failure through antiapoptotic action,[9] to ameliorate pulmonary emphysema,[10] to reverse chronic vocal scarring[11] and to promote therapeutic angiogenesis in a peripheral arterial disease model.[12] Recent phase I/II clinical studies in patients with some of those organ failures revealed no severe side effects of HGF treatment in humans.[13,14] However, the multiple biological activities of HGF do raise the possibility that undesirable effects might occur after systemic administration without careful control of the dose regimen. Therefore, it is essential to understand the overall pharmacokinetics of HGF in humans.

The various biological activities of HGF are thought to be primarily mediated by HGF receptor (c-Met protooncogene product).[1,2] This receptor is also proposed to be involved in systemic clearance of HGF. For example, after down-regulation, i.e., reduction of the receptor density on the cell surface, induced by the injection of a relatively high dose of HGF, the total body clearance of ^{125}I-HGF first decreased, but subsequently increased due to recruitment of c-Met/HGF receptor to the cell surface.[15] A series of pharmacokinetic studies has clarified aspects of the pharmacokinetics of HGF in rats: (i) the liver is the major clearance organ for HGF;[16-19] (ii) the pharmacokinetics of HGF is nonlinear due to the presence of two saturable elimination mechanisms;[18,19] (iii) the high- and low-affinity elimination mechanisms have been identified as receptor-mediated endocytosis (RME) via c-Met/HGF receptor and endocytosis via cell-surface heparin-like substance, respectively.[15-18] The molecular mechanism of the latter elimination pathway is still unknown, but this elimination may be mediated by heparan-sulfate proteoglycans, which are known to bind HGF.[20,21] However, despite the pharmacokinetic information obtained in experimental animals and humans, no physiologically based pharmacokinetic model for HGF has yet been developed. In addition, repetitive injection of HGF is usually needed to obtain pharmacological effects *in vivo* in experimental animals[3-9] and might be necessary in clinical application, but nevertheless, no data are available regarding the pharmacokinetic profile after repeated administration of HGF. Down-regulation of the HGF receptor/c-Met induced by HGF injection would affect systemic clearance after repeated doses.[15]

Considering the complexity of the pharmacokinetic properties of HGF, it seems essential to construct a mechanism-based pharmacokinetic model in experimental animals in order to understand in detail the disposition of HGF in humans.

Therefore, the purpose of the present study was to develop a pharmacokinetic model that can explain the plasma concentration profiles of HGF after a single dose and during constant infusion and repeated dosing, in order to evaluate possible species differences in the pharmacokinetics of HGF and to assist in the safe management of clinical trials. In particular, we focused on the following three points: (i) pharmacokinetic behavior of HGF in nonlinear dose condition; (ii) the effect of acute renal failure (ARF) on pharmacokinetics of HGF; (iii) the effect of repeated doses on the HGF pharmacokinetics, and possible down-regulation and subsequent recovery of the HGF receptor.

MATERIALS AND METHODS

Materials

Recombinant human HGF (5 amino acid-deleted type) was supplied by Kringle Pharma Inc. (Osaka, Japan). Glycerol and fluorescein-4-isocyanate (FITC) were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were commercial products of reagent grade.

Animals

Male ICR mice (25-35 g weight) were purchased from Japan Charles River Inc. (Yokohama, Japan) and maintained with free access to food and water. This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals at the Takara-machi Campus of Kanazawa University. To construct the acute renal failure (ARF) model, glycerol dissolved in saline (50% v/v) was injected at 9 mL/kg into the hind leg of each mouse. At 24 and 48 hr later, blood urea nitrogen (BUN) and alanine transaminase activity (ALT) in plasma were determined using the corresponding assay kits (Wako Pure Chemical) according to the manufacturer's instructions.

Synthesis of FITC-HGF

HGF (2 mg/mL) dissolved in 0.1 M sodium bicarbonate buffer (pH 9.0) was mixed with FITC (3 mg/mL) dissolved in DMSO and incubated at 4 ^{o}C for 8 hr, followed

by further incubation for 2 hr after addition of 1 M NH_4Cl. FITC-HGF was purified by gel filtration on a PD-10 column (Bio-rad, Hercules, CA) equilibrated with phosphate-buffered saline (PBS) containing 0.1% BSA, and stored at -80°C until use.

Pharmacokinetic Studies

Mice were fasted overnight with free access to water and anesthetized with diethylether during drug administration. HGF was diluted with 10 mM citrate buffer (pH 7.0) containing 0.3 M NaCl, 0.05% polysorbate 80, 1% sucrose, 5 mg/mL L-alanine and 0.2% BSA. HGF or cephalexin was then injected into the jugular vein, followed by blood sampling from the tail vein at 1, 5, 15, 30, 60, 240, 480, 720 and 1440 min for HGF and at 1, 5, 15, 30, 60 min for cephalexin. For intravenous infusion, mice were anesthetized with pentobarbital, and HGF was infused via the jugular vein with a basic syringe pump (KDS-101, BrainScience Idea Co. Ltd., Osaka, Japan) at 2.7 μL /min without any loading dose. Blood was sampled from the jugular vein on the other side at 5, 10, 20 and 30 min. The blood was rapidly mixed with 1.25 mg /mL of EDTA·2Na solution (1 : 4) and centrifuged at 3,000 g for 15 min to obtain plasma, which was stored at -80°C until use. The HGF concentration in plasma was measured using an enzyme immunoassay kit for human HGF (Institute of Immunology Co. Ltd., Tokyo, Japan). According to the previous report[22], this kit may almost exclusively detect intact HGF because the results obtained were well correlated to those obtained by western blot analysis and biological activity of

HGF. For the quantification of cephalexin, the plasma samples were mixed with methanol. All the mixed solutions were centrifuged at 15,000 rpm for 10 min at 4 °C. After the centrifugation, the supernatants were diluted with mobile phase (at a volume ratio of 1:1) and again centrifuged. The supernatants were then subjected to HPLC analysis. The HPLC system consisted of a constant-flow pump (LC-10Avp, Shimadzu, Kyoto, Japan), a UV detector (SPD-M10Avp, Shimadzu), an automatic sample injector (SIL-10A, Shimadzu), and an integrator (CLASS-VP, Shimadzu). The reversed-phase column (Cosmosil 5C18-AR-II, 4.6 x 150 mm; Nacalai Tesque) was maintained at 40˚C in a column oven (CTO-10Avp, Shimazu). The mobile phase was a mixture of 10 mM ammonium acetate and methanol (80:20), and the UV detector was set at 260 nm. The flow rate was 1.0 mL/min.

Tissue distribution of FITC-HGF

FITC-HGF (0.1 mg/kg) was injected into the jugular vein. At 11 min later, heparin (5,000 units/kg) was further injected to dissociate cell-surface-bound HGF.[17] At 15 min after the FITC-HGF injection, mice were transcardially perfused with phosphate-buffered saline (PBS) containing heparin (50 units/mL) and then 4% paraformaldehyde dissolved in 0.1 mM phosphate buffer (pH 7.4). The kidney and liver were removed and successively immersed in 4% PFA, 10, 20 and 30% sucrose/PBS. The tissue was then frozen and sectioned with a cryostat, and the sections (20 μm) were

mounted on glass slides. Fluorescence was detected with a confocal laser scanning fluorescence microscope (LSM 710, Carl Zeiss, Jena, Germany).

Immunostaining of human HGF

Frozen sections obtained as described above were used. For antigen activation, sections were heated to 92 ˚C in 20 mM Tris buffer (pH 9.0) for 15 min. Following successive pretreatment with 3% H_2O_2/PBS for 30 min, the sections were incubated with biotinylated anti-human HGF monoclonal antibody (Institute of Immunology Co. Ltd.) for 2 hr at room temperature and then washed with PBS. The immunoreaction product was visualized by incubating the sections successively with VECTASTAIN Elite ABC Standard Kit (Vector Laboratories) for 30 min and Peroxidase Substrate Kit including 3',3'-diaminobenzidine tetrahydrochloride (ImmPACT DAB, Vector Laboratories).

Construction of the pharmacokinetic model

The model is shown in Figure S1 in Supporting Information, and includes plasma and liver compartments connected via the plasma flow rate (Q_h). In the liver, HGF binds to two receptors, c-Met and heparin-like substance, followed by internalization of HGF-receptor complex with a rate constant k_{int} and subsequent degradation (Fig. S1). Unbound receptor is also internalized with a rate constant k_{deg} and recruited to the cell-surface at a rate of V_{syn} (Fig. S1). A mathematical model representing RME was

principally established by Wiley and Cunningham.[23] However, in the present study, considering that available information from clinical studies is limited, and only the plasma concentration profile can be fitted to the model, rapid equilibrium was assumed for the cell-surface binding of HGF to the receptors with a dissociation constant (K_d), to minimize the number of parameters. Mass-balance equations for plasma, liver and HGF receptors were, therefore, written as:

$$V_p(dC_p/dt) = - QC_p + QC_e + V_{syn,HGF} \quad (1)$$

$$dX_{liver}/dt = QC_p - QC_e - k_{int,1}(C_e R_{tot,1} /(K_{d,1} + C_e)) - k_{int,2}(C_e R_{tot,2} /(K_{d,2} + C_e)) - CL_{ns} C_p \quad (2)$$

$$dR_{tot,1}/dt = V_{syn,1} - k_{deg,1} (R_{tot,1} - (C_e R_{tot,1} /(K_{d,1} + C_e))) - k_{int,1} (C_e R_{tot,1} /(K_{d,1} + C_e)) \quad (3)$$

$$dR_{tot,2}/dt = V_{syn,2} - k_{deg,2} (R_{tot,2} - (C_e R_{tot,2} /(K_{d,2} + C_e))) - k_{int,2} (C_e R_{tot,2} /(K_{d,2} + C_e)) \quad (4)$$

where C_p and V_p are HGF concentration and distribution volume in the plasma compartment, respectively. X_{liver} and R_{tot} represent the amount of HGF and density of HGF receptor in the liver, respectively. As proposed in the previous report[23], we assumed steady-state condition in the absence of HGF

$$V_{syn} - R_{ss} k_{deg} = 0 \quad (5)$$

where R_{ss} represents receptor density at the steady-state before the injection of HGF and

was assumed to be the initial condition (receptor density at time zero) in pharmacokinetic analysis. According to Eq. (5), the k_{deg} can be written as V_{syn}/R_{ss}. The subscript numbers (1 and 2) represent heparin-like substance and c-Met, respectively. CL_{ns} is non-saturable clearance and was considered only in monkeys (see Results). $V_{syn,HGF}$ represents the biosynthesis rate of endogenous HGF and was considered in monkeys, since enzyme immunoassay for human HGF also recognizes monkey HGF. C_e represents HGF concentration in the extracellular space and can be written as:

$$V_eC_e^3 + (R_{tot,1} + R_{tot,2} + K_{d,1}V_e + K_{d,2}V_e - X_{liver})Ce^2 + (K_{d,1}R_{tot,2} + K_{d,2}R_{tot,1} + K_{d,1}K_{d,2}Ve - K_{d,1}X_{liver} - K_{d,2}X_{liver})C_e - K_{d,1}K_{d,2}X_{liver} = 0 \quad (6)$$

where V_e represents the distribution volume in extracellular space (see Supporting Information for details of the derivation of these equations).

Estimation of parameters

All the pharmacokinetic parameters were estimated based on the mean values of plasma concentration data. Pharmacokinetic data used for estimation of parameters were partly obtained in the present study in mice, and partly taken from previous studies in rats[18,19] and humans[24], and preclinical studies in cynomolgus monkeys (Kringle Pharma Inc.). Q_h was taken from the literature.[25] Dissociation constants $K_{d,1}$ and $K_{d,2}$ were taken to be fixed at 1615 and 2.62 ng/mL, respectively (assuming that the molecular weight of HGF is 82,000), for the association of HGF with heparin-like substance and c-Met,

respectively.[26,27] In case of a single dose, V_{syn} was assumed to be zero. During repeated dosing in monkeys, V_{syn} was written follows, assuming negative feedback by HGF as reported for c-Met:[28]

$$V_{syn} = V_{syn0} (1 - C_e / (IC_{50} + C_e)) \quad (7)$$

Here, the negative feedback was assumed to be mediated by the interaction of HGF with the corresponding receptor (c-Met and heparin-like substance), and therefore the IC_{50} was fixed as the K_d value for each receptor. V_{syn0} represents the zero-order rate constant. In rodents the other six parameters, $k_{int,1}$, $k_{int,2}$, $R_{tot,1}$, $R_{tot,1}$, V_p and V_p, were obtained by simultaneous nonlinear least-squares fitting of the model to the plasma concentration profiles at all doses examined after bolus injection and during infusion using the nonlinear regression analysis program Napp (Ver 2.0.1 for Macintosh OS-X, The University of Tokyo Hospital), while C_e was numerically calculated using the Newton-Raphson method. In monkeys and humans, the fitting was performed for intravenous bolus data.

RESULTS

Pharmacokinetics of HGF in normal and ARF mice

HGF was intravenously administered in mice at three different doses as a bolus (Fig. 1a) or infusion (Fig. 1b). The doses of the bolus injection were based on the amount of HGF which exhibited pharmacological effect in the previous reports (0.3 - 1 mg/kg; [3,29]). The infusion study was performed in addition to the bolus injection study with an aim to increase the plasma concentration data especially at lower range and support the goodness of the fitting in the pharmacokinetic modeling. This infusion was performed only for 30 min to minimize the externalization and subsequent recovery of HGF receptor during the infusion period. Disappearance of HGF after the single bolus dose was initially quite rapid, followed by a slower phase of disappearance in rodents (Fig. 1a). After the start of the infusion, the plasma HGF concentration rapidly increased and approached a plateau value (Fig. 1b).

Clinical development of HGF is now ongoing for treatment of ARF ahead of other diseases. Therefore, we next examined the effect of ARF on the pharmacokinetics of HGF with an aim to construct pharmacokinetic model in the ARF condition. Glycerol was intramuscularly injected in mice to construct the ARF model (Fig. 2a). At 24 hr after single injection of glycerol, both BUN and ALT in plasma was higher than those in the saline-treated group, whereas only BUN was significantly higher than the control at 48 hr (Fig. 2b, 2c), suggesting that the glycerol injection resulted in injury to both liver and

kidney at 24 hr, but the hepatic function had recovered by 48 hr. Therefore, mice at 48 hr after the glycerol injection were used as the ARF model, and the plasma concentration profile of HGF after intravenous injection was examined. The plasma concentration in the ARF model could be almost superimposed on that in control mice (Fig. 2a), suggesting a minimal effect of ARF on systemic clearance of HGF. Moreover, AUC/Dose and $T_{1/2}$ in ARF mice were similar to those in normal mice (Table 1). Therefore, we did not further consider the pharmacokinetic modeling of HGF in the ARF condition.

To examine the local events that may influence the pharmacokinetics of HGF, FITC-HGF was intravenously injected into normal and ARF mice, and fluorescence images were taken of frozen sections from kidney and liver (Fig. 3). In our preliminary study, a nonspecific signal was observed throughout the sections of both organs, probably because of efficient binding of HGF to cell-surface heparin-like substance. Therefore, heparin was also injected to remove such binding before the detection of fluorescence. FITC-HGF was detected in renal tubular epithelial cells in both normal and ARF mice (Fig. 3a, 3b, 3d, 3e). Compared with the normal kidney (Fig. 3a), the kidney sections from ARF mice more obviously showed tubular dilatation which was regarded as the damaged tubules according to the previous reports.[30] In kidney of ARF mice, fluorescence was clearly seen in those damaged tubules (Fig. 3d, 3e). Fluorescence was detected in vesicular structures of kidney in both normal and ARF mice (Fig. 3b, 3e), and further, the fluorescence inside hepatocytes was similar in normal and ARF mice (Fig. 3c, 3f). To confirm the specific endocytosis of

FITC-HGF via the HGF receptor, FITC-HGF with or without unlabeled HGF (30 mg/kg) was intravenously administered, and fluorescence in the kidney section was observed (Fig. 3g, 3h). The fluorescence was much reduced by the unlabeled HGF (Fig. 3g, 3h), suggesting receptor-mediated endocytosis of FITC-HGF. Additionally, to confirm localization of intact human HGF in the kidney, immunostaining using antibody for human HGF was performed in the kidney sections prepared from the mice administered with FITC-HGF. The immunostaining signal was detected in the kidney section, but reduced when the antibody was absorbed by its antigen (Fig. S2 in Supporting Information), suggesting that at least a part of FITC-HGF localized in the kidney was intact. To further support the damage of renal tubules in the ARF mice, we examined pharmacokinetics of cephalexin, which is known to be highly secreted in renal tubules. Plasma clearance of cephalexin assessed in different mice in the same ARF condition was much reduced (22% of that in control mice).

Model-independent and compartment analyses

Pharmacokinetic parameters in mice obtained by model-independent analysis of plasma concentration profiles were shown in Table 1. Steady-state distribution volume (Vd_{ss}), mean residence time (MRT) and half-life at terminal phase ($T_{1/2}$) increased as the increase in Dose (Table 1), indicating that elimination of HGF is delayed possibly because of saturation of elimination mechanism. On the other hand, the dose-normalized AUC

(AUC/Dose) and total clearance (CL_{tot}) were similar among all doses (Table 1). Plasma concentration profile at early phase (~60 min) was almost parallel at three doses (Fig. 1a, inset). In addition, the dose normalized AUC at the early phase ($AUC_{0\text{-}60}$/Dose) showed minimal difference among all doses and accounted for the major portion of total AUC ($AUC_{0\text{-}60}/AUC_{0\text{-}inf}$ > 74% for all the doses, Table 1). Thus, pharmacokinetics of HGF at the initial phase was almost linear, but that at terminal phase was nonlinear, suggesting the complex pharmacokinetic behavior of HGF.

Plasma concentration profile in mice after bolus injections (Fig. 1a) were next simultaneously fitted to the compartment models. The analysis using two-compartment model provided the best goodness of fit, Akaike's information criterion being the lowest than the other compartment models. Nevertheless, the fitting cannot fully explain overall plasma concentration profiles even after the single dose (Fig. 1a).

It should be noted that trough concentration was increased by the repeated doses (Fig. 4c, 4d, 4e, 4f), and this cannot be simply explained by the compartment model. HGF is known to be eliminated via at least two types of specific receptor.[15-18] Therefore, we next attempted to construct mechanism-based pharmacokinetic model including receptor-mediated endocytosis (Fig. S1) with an aim to understand pharmacokinetic mechanism.

Pharmacokinetic modeling of HGF in experimental animals and humans

First, plasma concentration profiles after bolus injection and during infusion in mice (Fig. 1) were simultaneously fitted to the model shown in Fig. S1. To minimize the number of parameters to be estimated in the fitting, the K_d values for c-Met and heparin-like substance were fixed at the reported values[26,27]. In addition, V_{syn} could not be determined by the fitting of single-dose data and therefore was fixed to be zero. Therefore, the parameters estimated by the fitting were V_p, V_e, $R_{ss,1}$, $k_{int,1}$, $R_{ss,2}$ and $k_{int,2}$. The results are listed in Table 2. The pharmacokinetic model reasonably well described the plasma concentration profiles in mice after bolus injection and during infusion (Fig. 1).

A similar fitting procedure was also performed for reported pharmacokinetic data[18,19] obtained in rats. The plasma concentration profiles after bolus injection and during infusion in rats were simultaneously fitted to the model, and the fitted lines coincided well with the data (Fig. 4a, 4b). The k_{int} values estimated by the fitting in rats (0.074 - 0.177 min^{-1}, Table 2) were higher than those in mice, but not very different from the reported value (~ 0.1 min^{-1}) for the internalization of HGF observed in perfused rat liver.[31]

In monkeys, preclinical data had been previously obtained after repeated intravenous injection of HGF at three different doses for 28 days (Fig. 4c, 4d, 4e, 4f). A similar fitting procedure to that used in the case of rodents was first performed for the data at day 1 (Fig. 4c), but the fitting was unsuccessful when the two K_d values were fixed, probably because the concentration range of HGF in monkeys appeared to be higher than in the other animals, and few plasma concentration data were available in to the region of the

reported Kd value for c-Met (2.62 ng/mL) in monkeys (Fig. 4c), leading to difficulty in estimation of kinetic parameters associated with c-Met ($R_{tot,2}$, $K_{d,2}$ and $k_{int,2}$) by fitting. Therefore, we fixed the values of these three parameters to be the same as those in rats, and then performed the fitting of the data at day 1 in monkeys. The fitting was successful when CL_{ns} was included in addition to c-Met and heparin-like substance. $V_{syn,HGF}$ was next estimated and fixed to yield an endogenous HGF concentration of 0.478 ng/mL, which was the mean value of plasma endogenous HGF obtained before the HGF injection in monkeys in the preclinical studies. Then, simultaneous fitting of all the data on days 1, 3, 14 and 28 at the three doses to the model was performed to estimate $R_{ss,2}$, $K_{d,2}$, CL_{ns}, $V_{syn,1}$ and $V_{syn,2}$, and this yielded the parameter values shown in Table 2. Here, the k_{deg} was assumed to be equal to V_{syn}/R_{ss} according to Eq. (5) and not directly estimated by the fitting. Thus, the pharmacokinetic model can describe the plasma concentration profile after repeated injections at all the doses examined (Figs. 4c, 4d, 4e). It should be noted that $K_{d,2}$ in monkey was estimated to be much higher than the reported K_d value[27] for the association of HGF with c-Met (Table 2).

The fitting procedure in humans was performed using the reported pharmacokinetic data after intravenous single administration[24]. In humans, the parameters for c-Met ($R_{ss,2}$, $K_{d,2}$ and $k_{int,2}$) could not be estimated because data at only one dose are available in the literature[24] (Fig. 4g). Therefore, the parameters for c-Met and $K_{d,1}$ were fixed at the same values as those in monkeys to estimate V_p, V_e, $R_{tot,1}$ and $k_{int,1}$ by fitting

(Table 2). Since only pharmacokinetic data after single administration were reported in humans, the fitting did not include CL_{ns} or $V_{syn,HGF}$. The fitted line coincided well with the data (Fig. 4g).

Overall, the V_p estimated by this fitting in rats and humans was close to the plasma volume (30~50 mL/kg)[25] and the reported value (52 mL/kg) obtained in rats,[16] but the values in mice and monkeys were higher (Table 2). The V_e in all species was much higher than the extracellular volume (10~20 mL/kg)[25] in the liver (Table 2).

DISCUSSION

HGF exhibits various pharmacological activities in experimental renal failure models and is currently under development as a therapeutic agent for kidney injury. However, there has been no information on its pharmacokinetics in renal failure. Therefore, we first compared the pharmacokinetics of HGF between normal and ARF mice. We found that the plasma concentration profile after intravenous injection was similar in the two groups (Fig. 2a), indicating that only a minimal change in systemic clearance of HGF occurs during renal failure. Intrahepatic distribution of FITC-HGF also exhibited only a minimal difference between the groups (Fig. 3c, 3f). Several pharmacokinetic studies in rats have indicated that liver is the major clearance organ for HGF.[16-19] Therefore, the minimal change in pharmacokinetics in the ARF model (Fig. 2a, Table 1) can be explained by the minor contribution of the kidney to systemic clearance. It is noteworthy, however, that renal handling of FITC-HGF exhibited some difference between normal mice and the ARF model: FITC-HGF was evenly distributed to renal tubules in control mice (Fig. 3a, 3b), whereas it was highly distributed to and internalized in damaged tubules in the ARF model (Fig. 3d, 3e). This may be compatible with the potent biological activities of HGF in renal tubules, such as anti-fibrosis, anti-apoptosis and stimulation of tubular proliferation.[3-5,7,32,33] In addition, the HGF distribution specifically to damaged tubules may be governed by similar mechanisms to those involved in proliferative activity of HGF and down-regulation of c-Met/HGF receptor in regenerating organs *in vivo*.[34,35] Thus, HGF

appears to be efficiently endocytosed in injured renal tubules, but such renal handling only minimally affects its overall clearance. HGF should be difficult to be filtered through glomerulus because of its large molecular size (> 80 kDa). Therefore, endocytosis of HGF should occur from basolateral membranes in normal kidney. However, structure of glomerulus could be damaged in ARF mice because of the increase in BUN (Fig. 2b). In addition, c-Met was reported to be localized on both basolateral and apical membranes.[36] Therefore, a part of HGF may be filtered and endocytosed from apical membranes in ARF condition.

Receptor-mediated endocytosis plays an important role in systemic clearance of various types of therapeutic proteins.[37-39] In addition, target-mediated disposition has been proposed for various types of therapeutic proteins and monoclonal antibodies.[40,41] In both cases, however, a single type of receptor/target is generally considered to be involved in systemic clearance. On the other hand, clearance of HGF has been suggested to be mediated by at least two binding sites, c-Met and cell-surface heparin-like substance.[16-18,31] Most of the data supporting the involvement of the two clearance sites were, however, obtained using systemically administered ^{125}I-labeled HGF as a tracer.[15-17,31] Thus, it has remained unclear whether the two sites are involved in HGF clearance over the same range of HGF concentrations. In the present study, our pharmacokinetic model assuming at least two binding sites in the liver could very well explain the plasma concentration profiles observed at various doses in mice, rats, monkeys and humans (Figs. 1, 4). Fitting was

successful at all the doses examined in the case of both bolus and infusion studies in rodents, and repeated doses in monkeys (Figs. 1, 4). These results suggest that systemic elimination of HGF is mediated by at least two clearance sites in the liver over a wide range of doses in various dose regimens. Nevertheless, pharmacokinetic information in humans is still quite limited, and therefore, kinetic parameters for only the low-affinity site were estimated by fitting of the plasma concentration profile (Fig. 4g). The difference in pharmacokinetics between human and mice may result from that in the affinity of human HGF to mouse and human c-Met. Nevertheless, the data in humans (Fig. 4g) include only plasma concentrations higher than 10 ng/mL, which would be enough to saturate the binding of HGF to c-Met, if we consider the dissociation constant of the interaction (~2.62 ng/mL).[27] This may bias the estimation of exact value for the affinity to c-Met in humans. Therefore, pharmacokinetic modeling in humans will need to be re-evaluated when further information becomes available at various doses. In addition, the $K_{d,2}$ in monkeys was much higher than the reported K_d value[27] for the association of HGF with c-Met (Table 2). Non-saturable elimination clearance (CL_{ns}) was obtained only in monkeys (Table 2). Although reasons for these phenomena remain to be established, it is noteworthy that the data in monkeys were obtained in a 28-day toxicity study, which included relatively high doses. In monkeys, the number of plasma concentration data close to the K_d value for c-Met (2.46 ng/mL) was quite limited; most of the data were obtained within the range of 10^2~10^5 ng/mL (Figs. 4c-4f). Therefore, the parameters obtained by the fitting in monkeys

may mainly represent those in a higher dose range than the clinically relevant one.

The terminal phase after bolus injection exhibited nonlinear behavior in mice, the half-life and MRT being prolonged at higher doses (Fig. 1a, Table 1). This was also the case in rats and monkeys (Fig. 4) and can be explained by a nonlinear elimination mechanism such as RME. In addition, such a prolonged half-life at higher doses was observed even for the same plasma concentration range: for example, in the plasma concentration range of 10-100 ng/mL, the elimination curve was more gradual at 21.7 mg/kg compared with those at 0.72 and 2.33 mg/kg in mice (Fig. 1a). A similar phenomenon was found in rats and monkeys (Fig. 4). In addition, at higher plasma concentration of HGF (10^4~10^5 ng/mL), the elimination at the highest dose was slightly slower than that at lower doses (Fig. 1a, inset). These results cannot be simply explained by nonlinear elimination and may indicate exposure-dependent elimination kinetics. Such unique kinetics may be inconsistent with the application of a simple Michaelis-Menten type kinetic model to the disposition of HGF. The pharmacokinetic model proposed in the present study includes endocytosis of HGF-receptor complex at both high- and low-affinity sites. Therefore, higher exposure of HGF to c-Met and/or heparin-like substance leads to a decrease in receptor density (down-regulation) on the cell surface, resulting in a delay of systemic elimination. The fitted lines obtained for the data after single bolus administration fairly well explain such elimination profiles in all the animals (Figs 1, 4), supporting the validity of the RME model proposed in the present study.

The model-independent analysis revealed unique characteristics in pharmacokinetics of HGF (Table 1). Vd_{ss}, MRT and $T_{1/2}$ were dose-dependent whereas AUC/Dose and CL_{tot} were dose-independent (Table 1). When we observed details in plasma concentration profile (Fig. 1a), nonlinear behavior was observed primarily at the terminal phase: The elimination curve at the terminal phase was more gradual at the higher doses (Fig. 1a), leading to the prolonged $T_{1/2}$ and MRT, and larger Vd_{ss} (Table 1). On the other hand, plasma concentration profile at the initial phase showed minimal nonlinear behavior. Actually, the $AUC_{0\text{-}60}$/Dose was almost close among all the doses and accounted for a large part of AUC until infinite time (Table 1). Thus, even when other parameters like Vd_{ss}, MRT and $T_{1/2}$ exhibited dose-dependence, AUC/Dose and CL_{tot} were apparently dose-independent probably because they were largely affected by the plasma profile at the initial phase. The mechanism for nonlinear behavior especially at the terminal phase was still unknown, but can be accounted for by the down-regulation of HGF receptors due to the long-time exposure to HGF according to the present pharmacokinetic model.

HGF receptor density on the cell surface gradually recovers after endocytosis induced by HGF. Indeed, Liu et al.[15] kinetically analyzed the recovery of c-Met in rats *in vivo* and obtained a half-life of 1~3 h for the recovery in various organs. The pharmacokinetic model in the present study includes the recruitment of both c-Met and heparin-like substance to the cell surface. Nevertheless, the pharmacokinetic profile after single intravenous bolus administration and during short-term (30~90 min) infusion could

be explained even when V_{syn} was set to be zero (see Results), suggesting that recovery of the HGF receptors may only minimally affect pharmacokinetics of HGF after a single dose. On the other hand, repeated injection of HGF in monkeys caused a gradual increase in trough values (plasma HGF concentration at 1,440 min in Fig. 4c-4f). The increase in the trough values was marked at higher dose (30 mg/kg), but was also apparent at the lowest dose (0.3 mg/kg, Fig. 4c-4f). There are at least two possible explanations for such a phenomenon. One is the induction of biosynthesis of HGF in the body, and the other is a decrease in recruitment of HGF receptors after repeated injections. The injection of HGF and plasmid encoding HGF gene are known to induce biosynthesis of HGF *in vivo*,[3,42] supporting the former possibility. However, the enzyme immunoassay used in the monkey studies cannot distinguish endogenous (monkey) HGF from human HGF exogenously administered, and therefore, we cannot identify the contribution of endogenous HGF to the increase in the trough values. The trough concentration of HGF at the highest dose (30 mg/kg) was 10^2~10^4 ng/mL (Figs. 4c-4f). These values are very much (10^3~10^5 times) higher than the endogenous plasma concentration. On the other hand, Mizuno et al.[3] reported that HGF supplement induced an increase in the endogenous HGF level in kidney in mice, but the increase was less than two-fold versus the saline control. Ueki et al.[42] reported an increase in endogenous HGF level in circulating plasma after transduction with the HGF gene, but the increase was at most four-fold versus the control. Thus, it seems unlikely that induction of biosynthesis of endogenous HGF can fully explain the increase in

the trough values. On the other hand, Hammond et al. reported that HGF induced degradation of c-Met, leading to a decrease in the protein level.[28] This supports the latter possibility, i.e., a gradual decrease in recruitment of HGF receptors to the cell surface during exposure to HGF. Such a decrease in recruitment of HGF receptors to the cell surface causes another type of down-regulation (a decrease in receptor density), which is different from that induced simply by endocytosis of HGF-receptor complex. We incorporated such a decrease in recruitment of HGF receptors during repeated doses of HGF into our model, and this assumption could at least partially explain the increase in the trough concentration (Fig. 4c-4f). Thus, recruitment of HGF receptors may affect the pharmacokinetics after repeated doses, although further studies are needed to clarify the precise mechanism(s) involved.

After intravenous bolus injections, HGF initially disappears with a quite short half-life in plasma (~ 4 min).[16] This was also observed in the present study in animals and human (Figs. 1, 4). The rapid elimination was apparent within a short period after injection (~15 min), and there was a more than two-order-of-magnitude decrease in plasma HGF concentration, followed by a slower elimination phase (Figs. 1, 4). Such a rapid and transient decrease at the initial phase after intravenous bolus injection can be explained by the rapid association of HGF with cell-surface binding sites. Actually, after intravenous administration of ^{125}I-HGF, tissue uptake clearance of the isotope at the initial phase (1~ 5 min) was much higher in the liver, compared with other organs.[16] Therefore, in the present

study, we assumed the presence of two cell-surface receptors in the liver compartment (Fig. S1). Nevertheless, the fitting procedure yielded V_e values much higher than the physiological values (Table 2), indicating rapid association of HGF with other binding sites in the extracellular space. Various types of binding sites have been proposed for HGF. For example, HGF binds not only to heparan-sulfate proteoglycans,[43] but also to various other types of glycosaminoglycans, such as dermatan sulfate.[26] c-Met is expressed not only in hepatocytes, but also in endothelial cells.[44] Further work will be needed to clarify the molecular mechanisms involved in such extremely high V_e.

Distribution of FITC-HGF into the kidney in ARF seems to be different from that in normal condition (Fig. 3a, 3d) whereas the pharmacokinetic model did not include the kidney (Fig. S1). This was because the pharmacokinetic model constructed in the present study was aimed to simply describe the systemic concentration of HGF. The liver is the major clearance organ for HGF[16-19], and HGF is largely distributed to the liver compared with other organs including kidney.[16,45,46] Kato et al[19] directly estimated renal clearance of HGF and found its minimal contribution to systemic clearance in rats. The minimal contribution of the kidney to systemic clearance could be compatible with the present finding that plasma concentration profile in ARF mice was similar to that in normal mice (Fig. 2a). Thus, the pharmacokinetic model did not include the kidney compartment (Fig. S1). Nevertheless, we still have to consider the possible relevance of the renal handling of HGF to its potential biological activity in renal tubules. Thus, the distribution kinetics of

HGF in the kidney may have to be considered to construct pharmacodynamics model which should be constructed by further analyses.

In conclusion, ARF may not affect systemic clearance of HGF, though internalization of HGF occurs efficiently in damaged renal epithelial cells. Two clearance mechanisms can principally account for the pharmacokinetic profiles of HGF in various dose ranges. Recruitment of HGF receptors minimally affects the pharmacokinetics of HGF after single dosing, but could account for the increase in trough values after repeated dosing. The pharmacokinetic model proposed in the present study is expected to contribute to the safe and efficient management of further clinical studies for the development of HGF as a therapeutic agent.

ACKNOWLEDGEMENT

We thank Ms Lica Ishida for technical assistance. We also thank Mr. Ryuichi Harada for performing immunostaing analysis and Mr. Shinya Uwafuji for performing a part of the fitting analyses. We also thank Dr. Akihiro Hisaka in The University of Tokyo Hospital for kind guidance on fitting procedure in Napp program.

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FOOTNOTES

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

LEGENDS TO FIGURES

Fig. 1 Plasma concentration profile of HGF and fitted curves obtained with the pharmacokinetic model after intravenous bolus injection and during intravenous infusion in mice

HGF was injected at a dose of 0.72 (△), 2.33 (◇) or 21.7 (○) mg/kg as a bolus in (a) and infused at a dose of 100, 300 or 1,200 ng/min/kg in (b). Plasma concentration was determined by enzyme immunoassay. Data within a short time period are also shown in the inset. Data represent mean ± SEM (n =3 - 4). Dotted lines represent fitted curves derived from two-compartment analysis. Straight li~~L i~~nes represent fitted curves derived from the pharmacokinetic model.

Fig. 2 Effect of ARF on pharmacokinetics of HGF in mice

The ARF model was constructed in mice by intramuscular injection of glycerol. Saline was intramuscularly injected as a control. HGF was intravenously injected at a dose of 2.33 mg/kg at 48 hr after the glycerol injection. Panel (a) Plasma concentration, determined by enzyme immunoassay, in normal (◇) and ARF (◆) mice. Panel (b) BUN level in plasma, determined at 24 hr after glycerol injection in normal (open bars) and ARF (closed bars) mice. Panel (c) ALT level in plasma, determined at 48 hr after glycerol injection in normal (open bars) and ARF (closed bars) mice. Data represent mean ± SEM (n =7 - 12).

*, Significantly different from saline-treated group ($p < 0.05$)

Fig. 3 FITC-HGF was internalized into renal tubular cells in normal and ARF mice

Fluorescence was detected using confocal microscopy in frozen tissue sections obtained from kidney (a, b, d, e) and liver (c, f) at 15 min after intravenous injection of FITC-HGF (2.01 mg/kg) in normal (a, b, c) and ARF model (d, e, f) mice. In panels (g) and (h), FITC-HGF with (h) or without (g) unlabeled HGF (30 mg/kg) was administered, and fluorescence was detected using confocal microscopy on the same gain setting for comparison between each panel. Note that fluorescence was detected in damaged tubular structures showing tubular dilatation (arrowheads) in panels (d, e).

Fig. 4 Plasma concentration profiles of HGF and fitted curves obtained with the pharmacokinetic model in rats, monkeys and humans

Plasma concentration in rats after intravenous bolus injection (panel a) at 0.1 (×), 0.3 (—), 1 (∗), 3 (□), 10 (■), or 30 (△), 120 (▲), 300 (◇), 500 (◆), 710 (○) or 1,000 (●) μg/kg and during intravenous infusion (b) at 0.0133 (△), 0.0310 (▲), 0.0703 (◇), 0.230 (◆), 1.07 (○), or 5.77 (●) μg/min/kg were taken from the literature.[18,19] Data within a short time period are also shown in the inset of panel (a). Plasma concentration in monkeys after repeated intravenous injection (once per daily) at 0.3 (△), 3 (◇) or 30 (○) mg/kg at day 1 (c), day 3 (d), day 14 (e) and day 28 (f) was taken from an unpublished

preclinical study (Kringle Pharma Inc.). Note that the trough values (at 1,440 min in each panel) gradually increased during repeated dosing. The value of 1.43 mg/kg in humans with chronic liver disease (g) was taken from the literature.[23] Lines represent fitted curves derived from the pharmacokinetic model.