# Effective Administration Route for the Deleted Form of Hepatocyte Growth Factor To Exert Its Pharmacological Effects

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**Abstract** □ The pharmacokinetics and the pharmacological effects of the deleted form of hepatocyte growth factor (dHGF) after intravenous (iv), subcutaneous (sc), or intramuscular (im) administration (0.25 and 2.5 mg/kg) were studied in rats. After single iv administration (2.5 mg/kg), dHGF in serum rapidly decreased ($\alpha$- and $\beta$-phase half-life: 3.2 and 26.5 min, respectively). Two to four hours after single sc or im administration (2.5 mg/kg), the serum level of dHGF reached a maximum and then gradually declined (half-life: 2.7 h). The serum levels were not changed by repetitive iv administration, but were dramatically decreased by repetitive sc or im administration. Liver weight and serum levels of total protein, albumin, and HDL-cholesterol were significantly increased by iv administration of dHGF (twice daily for 4 days at 0.25 mg/kg). Sc or im administration of dHGF did not increase these parameters at the same dose, but did significantly at 2.5 mg/kg. These observations suggest that iv administration is the most effective in exerting the pharmacological effects of dHGF among three administration routes. dHGF after iv administration was distributed mainly and rapidly into liver (53.6% of the injected dHGF within 5 min) and was sustained at a higher level in the liver than in plasma. In infusion (0.5 mg/kg/3 h), dHGF level in plasma and liver reached a steady-state 15 and 60 min after starting the infusion, respectively. The steady-state level of dHGF was 7- to 9-fold higher in liver than in plasma, and the higher level in liver was sustained beyond the steady-state.

Human hepatocyte growth factor (HGF) was purified and characterized as a mitogen for adult rat hepatocytes.[1,2] Two different complementary DNAs (cDNAs) encoding human HGF were cloned from human liver[3] and placenta[4] cDNA libraries. A major variant of HGF, the deleted form of HGF (dHGF), has been purified from conditioned medium of human fibroblasts.[5] dHGF cDNA, which lacks 15 nucleotides encoding a five-amino acid residue in the first kringle domain of HGF, has been isolated from human fibroblast cDNA library.[6] Like HGF, dHGF is a heparin-binding basic protein with an apparent molecular mass ($M_r$) of 76−80 kD and is a heterodimer composed of a large $\alpha$-chain with a $M_r$ of 52−56 kD and a small $\beta$-chain with a $M_r$ of 30−34 kD.[6] dHGF is more mitogenic than HGF for adult rat hepatocytes[6−8] and epithelial cells.[7] HGF and dHGF are distinguishable in their target cell specificity in growth stimulation and in their tertiary structure.[7] Serum HGF or dHGF levels in patients with liver diseases are higher than those in healthy subjects.[9,10] These observations suggest that dHGF acts on hepatocytes and plays an important role in liver regeneration.

To determine the most suitable administration route of dHGF, we investigated pharmacokinetics of dHGF after intravenous (iv), subcutaneous (sc), or intramuscular (im) administration and compared the effects of dHGF on hepatic protein synthesis, hepatic cholesterol metabolism, and liver weight among three routes of administration. dHGF exerted the most potent accelerating effect on liver functions and on increase of liver weight after iv administration. We also examined distribution of dHGF into the liver after iv administration. Exogenous dHGF was distributed mainly and rapidly into the liver and was sustained at higher concentrations in the liver than in plasma.

## Experimental Section

**Animals**—Male Wistar rats were obtained from Charles River Japan (Kanagawa, Japan). The animals were housed for at least 1 week before the study at 23 ± 2 °C under a 12 h light−dark cycle. Water and laboratory chow were provided ad libitum. All animals used were 7 weeks old and weighed between 185 and 245 g.

**Reagents**—dHGF was purified from the conditioned medium of Namalwa cells transfected with an expression plasmid comprising human dHGF cDNA.[7] The purified dHGF was diluted with sterile phosphate-buffered saline (PBS) containing 0.01% Tween 80. All other reagents were of analytical grade and were commercially available.

**Preparation of Anti-dHGF IgG**—Female Japanese White rabbits weighing about 2 kg received sc injections of dHGF (100 $\mu$g/rabbit) with complete Freund's adjuvant six times at intervals of 2 weeks, and blood was collected from the animals 1 week after the last injection. Anti-dHGF serum was subjected to protein A column (Nippon Bio-Rad Laboratories, Tokyo, Japan) chromatography, and the purified anti-dHGF IgG was stored at −20 °C. The concentration of anti-dHGF IgG was determined by the method of Lowry,[11] using bovine IgG as a standard protein.

**Preparation of Anti-dHGF IgG-Peroxidase Conjugate**—The anti-dHGF IgG was further purified by affinity chromatography on an Affigel 10 column (Nippon Bio-Rad Laboratories) coupled with dHGF. The purified anti-dHGF specific antibody (IgG) was conjugated to horseradish peroxidase using a ImmunoPure Maleimid Activated Horseradish Peroxidase kit (Pierce, Rockford, IL). The anti-dHGF IgG-peroxidase conjugate was supplemented with the same volume of glycerol and stored at −20 °C.

**Collection of Serum Samples for Pharmacokinetic Study**—In single administration, dHGF (0.25 or 2.5 mg/kg) was administered to rats through the tail vein (iv injection), dorsimesal subcutis (sc injection), or hind limb (im injection) of animals. In repetitive administration, dHGF (0.25 and 2.5 mg /kg/injection) was administered twice daily for 4 days to rats by iv, sc, or im injection as described above. Under ether anesthesia, blood was collected in a polypropylene tube through a glass capillary tube inserted into the orbital vein of the animals from 5 min to 24 h after iv injection and from 30 min to 24 h after sc or im injection. The blood samples were allowed to clot at room temperature and were centrifuged at 1300*g* for 10 min to separate serum. Each serum sample was transferred into a polypropylene microtube and was stored at −80 °C until used for dHGF assay.

**Analysis of Liver Functions**—dHGF (0.25 or 2.5 mg/kg) or vehicle was given to rats twice daily for 4 days by iv, sc, or im

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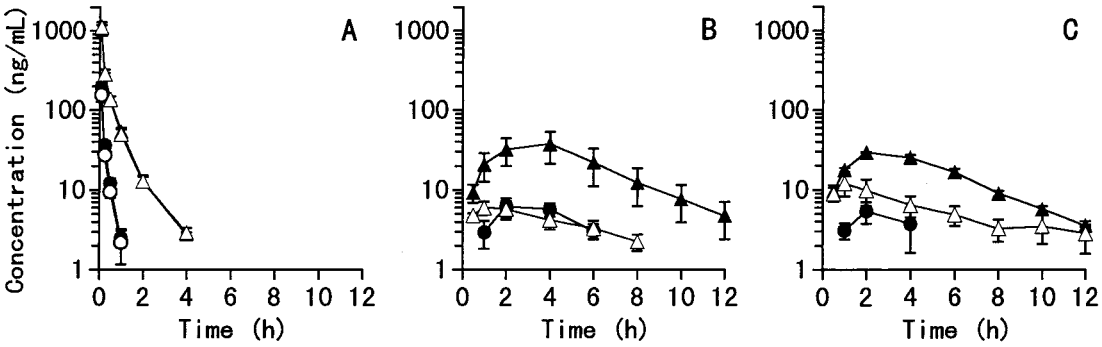


**Figure 1**—The concentration–time profiles of dHGF in serum after single and repetitive (A) iv, (B) sc and (C) im administrations of 0.25 and 2.5 mg/kg dHGF to rats. Key: single administration of 0.25 (●) and 2.5 (▲) mg/kg; repetitive administration of 0.25 (○) and 2.5 (△) mg/kg. Each point and bar represents the mean ± SD ($n = 3$). The concentrations of dHGF in serum after repetitive sc and im administrations of dHGF at a dose of 0.25 mg/kg were undetectable level (<1.25 ng/mL) in the greater part of the sampling time points.

injection. Under ether anesthesia 12 h after the last administration, blood was sampled from the inferior vena cava of the animals using a vacuum tube (Terumo, Tokyo, Japan). The blood sample was treated to separate serum as described above. The liver of each animal was excised for weighing at the same time. Serum samples were stored at −80 °C until used for assay. The concentrations of total protein, albumin, and HDL-cholesterol in serum were determined with a type 7150 automatic analyzer (Hitachi, Tokyo, Japan), using commercially available test reagents (Daiichi Pure Chemicals, Tokyo, Japan).

**Collection of Plasma and Liver Samples for Distribution Study**—The experiment on distribution of dHGF after iv bolus administration was carried out as follows. dHGF (0.5 mg/kg) was administered to rats through the tail vein. Under ether anesthesia, blood was collected from the inferior vena cava of the animals from 2 to 120 min after the administration using a vacuum tube containing EDTA. The liver was removed after perfusion with saline at the same time. The collected blood samples were centrifuged at 1300*g* for 10 min, and the obtained plasma samples were stored at −80 °C until used for dHGF assay. The removed liver was homogenized in 20 mM Tris-HCl (pH 7.5) containing 2 M NaCl, 0.1% Tween 80, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA with 4-fold volume of liver weight. The homogenate was centrifuged at 19000*g* for 30 min at 4 °C, and the resultant supernatant (liver extract) was collected and stored at −80 °C until used for dHGF assay. In addition, dHGF was administered to other rats at the same dose, and plasma and liver samples were collected up to 6 h after the administration as described above.

The experiment on distribution of dHGF after iv infusion was performed as follows. Under ether anesthesia, the right jugular vein was cannulated with polyethylene tubing (SP31, Natume, Tokyo, Japan). After recovery from anesthesia, iv infusion of dHGF solution through the tubing was performed at a flow rate of 2.1 μL/min (0.5 mg dHGF/kg/3 h) using a microsyringe pump (EP-60, Eicom, Kyoto, Japan). Under pentobarbital anesthesia, blood was collected and the liver was taken out from 15 min to 3 h after starting the infusion and from 15 min to 3 h after terminating the infusion, and these samples were treated and stored as described above.

**Enzyme-Linked Immunosorbent Assay for dHGF**—dHGF concentrations in serum, plasma, and liver extract were measured by a two-step sandwich enzyme-linked immunosorbent assay (ELISA) using rabbit anti-dHGF polyclonal antibody, which has no cross-reactivity to rat HGF.

ELISA for dHGF in serum or in plasma was performed as follows. Anti-dHGF IgG was diluted with 0.1 M $NaHCO_3$ to give a final concentration of 10 μg/mL. The diluted IgG solution (100 μL) was added to each well in 96-well immunoplates (MaxiSorp, InterMed, Roskilde, Denmark). The plates were allowed to stand at room temperature overnight. Subsequently, nonspecific binding sites of each well in the plates were blocked with 200 μL of Block Ace (Snow Brand Milk Products Co., Ltd., Tokyo, Japan) diluted 2-fold with water at room temperature for 1 h. After washing each well three times with PBS containing 0.1% Tween 20 (PBS-Tween), 50 μL of the first reaction buffer (50% Block Ace, 0.2 M NaCl, 0.1% Tween 20, 0.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 20 mM benzamidine hydrochloride, 10 mM EDTA in 0.2 M Tris-HCl, pH 7.3) was added to each well.

**Table 1—Pharmacokinetic Parameters of dHGF after iv, sc, and im Administrations in Rats**[a]

| pharmacokinetic parameter | route of admin | single admin 0.25 mg/kg | single admin 2.5 mg/kg | repetitive admin 0.25 mg/kg | repetitive admin 2.5 mg/kg |
|---|---|---|---|---|---|
| $AUC_{last}$, ng·h/mL | iv | 37.9 ± 3.4 | 302.5 ± 20.7 | 29.6 ± 3.4 | 309.9 ± 42.2 |
| | sc | 26.7 ± 6.2 | 230.9 ± 100.8 | *b* | 32.8 ± 7.0 |
| | im | 14.9 ± 4.7 | 177.8 ± 8.6 | *b* | 66.9 ± 20.4 |
| *F*, % | sc | 70.6 | 76.3 | *b* | 10.6 |
| | im | 39.3 | 58.8 | *b* | 21.6 |

[a] Each value is the mean ± SD of three animals. [b] Not calculated.

Then 50 μL of each serum (or plasma) sample or each dHGF standard prepared in normal rat serum (or plasma) was added to each well in the plates, and the plates were allowed to stand at 4 °C overnight. After washing each well three times with PBS-Tween, 100 μL of anti-dHGF IgG-peroxidase conjugate diluted 100-fold with the second reaction buffer (10% Block Ace, 0.15 M NaCl, 0.1% Tween 20 and 4% rat serum in 0.1 M phosphate buffer, pH 7.0) was added to each well, and the plates were incubated at 37 °C for 2 h. After washing each well six times with PBS-Tween, 100 μL of substrate solution (0.4 mg/mL of *o*-phenylenediamine dihydrochloride and 0.006% $H_2O_2$ in 0.1 M citrate−phosphate buffer, pH 4.5) was added to each well in the plates, and the plates were incubated at 37 °C for 30 min. The enzymatic reaction was terminated by addition of 50 μL of 6 N $H_2SO_4$. Absorbance at 492 nm was read using a microplate reader (MTP-32, Korona, Ibaragi, Japan).

To determine dHGF in liver extract, the ELISA system was modified slightly as follows. Here, 50 μL of liver extract in place of serum (or plasma) or 50 μL of dHGF standard prepared in liver extract derived from normal rats was added to each well containing 50 μL of the first reaction buffer. The plates were incubated at room temperature for 2 h. After washing the plates, 100 μL of anti-dHGF IgG-peroxidase conjugate diluted 200-fold with the second reaction buffer was added to each well in the plates, and the plates were incubated at room temperature for 2 h. After washing, bound peroxidase was assayed as described above.

**Pharmacokinetic Analysis**—The half-life in iv administration of dHGF (2.5 mg/kg) was calculated by fitting a biexponential equation using a nonlinear least-squares program of WinNonlin (Scientific Consulting Inc., Apex, NC). The terminal elimination half-lives in sc and im administrations (2.5 mg/kg), and the area under the concentration versus time curve up to the last measured time point ($AUC_{last}$) was calculated by using noncompartmental analysis program of WinNonlin. The bioavailability (*F*) was calculated by comparison of the $AUC_{last}$ after sc or im administration with that after iv administration.

**Statistical Analysis**—The pharmacological data were analyzed by analysis of variance followed by Fisher's LSD test. Significance was established at $p < 0.05$ or $p < 0.01$. The values are expressed as the mean ± SD, unless otherwise noted.

**Table 2—Effect of dHGF on Liver Weight and Serum Levels of Proteins and Cholesterol in Normal Rats[a]**

| route of admin | dose (mg/kg/injection) | liver weight (g/100 g body weight) | total protein (g/100 mL) | albumin (g/100 mL) | HDL-cholesterol (mg/100 mL) |
|---|---|---|---|---|---|
| iv | control | 3.34 ± 0.04 | 5.6 ± 0.1 | 2.7 ± 0.0 | 38.8 ± 2.2 |
| | 0.25 | 3.71 ± 0.13[b] | 6.4 ± 0.1[c] | 3.1 ± 0.0[c] | 72.0 ± 4.0[c] |
| | 2.5 | 4.30 ± 0.17[c] | 7.0 ± 0.1[c] | 3.3 ± 0.1[c] | 99.9 ± 6.9[c] |
| sc | control | 3.44 ± 0.10 | 5.4 ± 0.0 | 2.6 ± 0.0 | 37.5 ± 2.0 |
| | 0.25 | 3.28 ± 0.09 | 5.6 ± 0.1 | 2.8 ± 0.1 | 44.6 ± 1.5 |
| | 2.5 | 4.33 ± 0.14[c] | 6.9 ± 0.1[c] | 3.4 ± 0.1[c] | 99.4 ± 7.8[c] |
| im | control | 3.34 ± 0.10 | 5.6 ± 0.1 | 2.7 ± 0.1 | 37.4 ± 1.0 |
| | 0.25 | 3.34 ± 0.06 | 5.7 ± 0.1 | 2.8 ± 0.0 | 39.8 ± 3.9 |
| | 2.5 | 4.08 ± 0.09[c] | 6.8 ± 0.1[c] | 3.3 ± 0.0[c] | 83.1 ± 4.0[c] |

[a] Measurements were made after eight administrations (twice daily for 4 days) of dHGF. Each value represents the mean ± SEM of five animals. [b] $p < 0.05$ vs control (solvent) of the same route. [c] $p < 0.01$ vs control (solvent) of the same route.

## Results

**Pharmacokinetics of dHGF**—The concentrations of dHGF in serum decreased rapidly within 15 min and reached an undetectable level (<1.25 ng/mL) 2 to 8 h after a single iv administration of dHGF (0.25 and 2.5 mg/kg) to rats (Figure 1A). The profiles of dHGF level in serum after repetitive iv administration (twice daily for 4 days) of dHGF at the same doses were similar to the dHGF-elimination curves after single iv administration (Figure 1A). dHGF in serum after single and repetitive iv administrations (2.5 mg/kg) decreased biexponentially with a half-life of 3.2 min in the α-phase and with a half-life of 25.7 to 26.5 min in the $\beta$-phase. Single and repetitive iv administrations of dHGF at a dose of 2.5 mg/kg were about 10-fold larger in $AUC_{last}$ value than those at a dose of 0.25 mg/kg (Table 1).

The concentration−time profiles of dHGF in serum after single sc and im administrations of dHGF (0.25 and 2.5 mg/kg) were similar (Figure 1, parts B and C). In both single administrations (2.5 mg/kg), the concentrations of dHGF in serum reached a maximum after protracted absorption, which continued at least for 2 to 4 h after the injections and then decreased with a half-life of 2.7 h. The maximum level of dHGF in serum after sc or im administration was lower than after iv administration. On the other hand, the concentrations of dHGF beyond 1 or 2 h after administration were sustained at higher levels for sc and im administrations than for iv administration (Figure 1, parts A−C). The bioavailabilities of dHGF after single sc and im administrations were 70.6−76.3% and 39.3−58.8%, respectively (Table 1).

The concentrations of dHGF in serum after repetitive sc and im administrations (twice daily for 4 days) at a dose of 0.25 mg/kg were undetectable level (<1.25 ng/mL) in the greater part of the sampling time points. The serum levels and the $AUC_{last}$ values of dHGF in sc and im administrations of dHGF (2.5 mg/kg) were dramatically decreased by repeating their administrations (Figure 1, parts B and C, Table 1). The $AUC_{last}$ values of dHGF in repetitive sc and im administrations at a dose of 2.5 mg/kg were decreased to 14.2 and 37.6% of those in single sc and im administrations at the same dose, respectively, and were almost comparable to those in single and repetitive iv administrations at a dose of 0.25 mg/kg.

**Effect of Administration Routes of dHGF on Liver Weight and Functions**—Rats were treated by repetitive iv, sc or im administration of dHGF (twice daily for 4 days at 0.25 and 2.5 mg/kg). Serum levels of total protein, albumin, HDL-cholesterol, and liver weight after the administration are shown in Table 2. These parameters were significantly increased by repetitive iv administration of dHGF at a dose of 0.25 mg/kg, but not by repetitive sc or im administration at the same dose. However, repetitive sc or im administration significantly increased these parameters at a dose of 2.5 mg/kg.

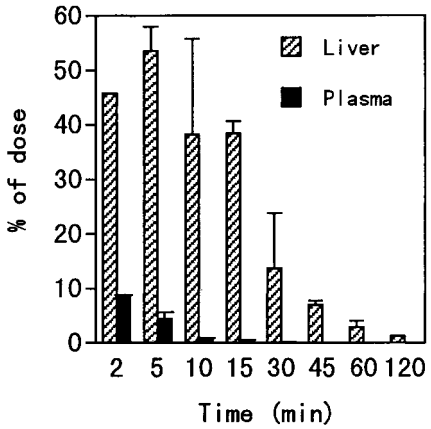


**Figure 2**—Distribution of dHGF in liver and plasma after iv bolus administration (0.5 mg/kg) to rats. Each value represents the mean ± SD ($n$ = 2–3).

**Distribution of dHGF into Liver**—After iv bolus injection of dHGF (0.5 mg/kg) to rats, 53.6 ± 4.4% of the injected dHGF was distributed into liver within 5 min, and 1.2 ± 0.1% of the injected dHGF remained in liver up to 120 min (Figure 2). dHGF distributed in plasma 2 min after iv injection was only 8.8% of the injected dHGF (Figure 2). The concentration−time profiles of dHGF in plasma and liver up to 6 h after iv bolus injection (0.5 mg/kg) are shown in Figure 3A. The concentrations of dHGF decreased more slowly and were sustained at higher level in the liver than in plasma. The ratios of the dHGF level in liver to that in plasma after iv injection ranged from 4 to 166.

When dHGF was infused to rats at a rate of 0.5 mg/kg/3 h, the dHGF level in plasma reached a steady-state 15 min after starting the infusion and declined rapidly after terminating the infusion (Figure 3B). The steady-state level of dHGF in plasma ranged from 53.7 ± 10.0 to 76.0 ± 22.1 ng/mL. The dHGF level in the liver reached a steady-state 60 min after starting the infusion and declined more slowly than did that in plasma after terminating the infusion (Figure 3B). The steady-state level of dHGF in the liver was 7- to 9-fold higher than that in plasma and ranged from 486 ± 59 to 551 ± 51 ng/g liver.

## Discussion

The concentrations of dHGF in serum after single iv administration of dHGF (2.5 mg/kg) decreased rapidly with an initial half-life ($t_{1/2}\alpha$) of 3.2 min. The initial half-life data reported here are consistent with the result on native dHGF purified from human embryonic fibroblasts[12] and with those on recombinant human HGF.[13−16]

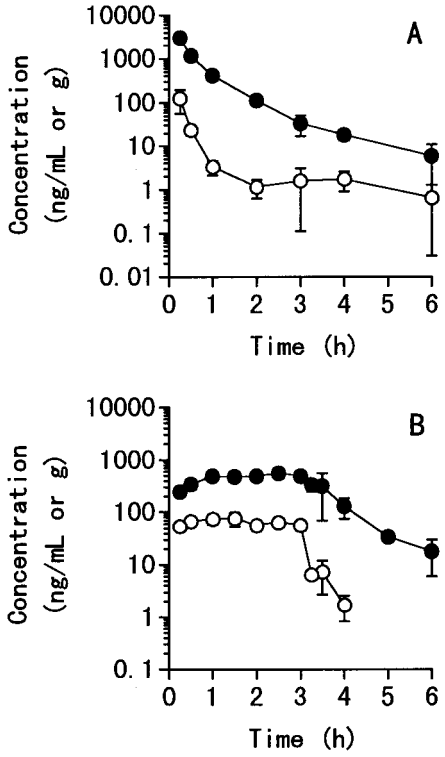


**Figure 3**—The concentration–time profiles of dHGF in plasma (○) and liver (●) after (A) iv bolus administration (0.5 mg/kg) and (B) iv infusion (0.5 mg/kg/3 h) to rats. Each point and bar represents the mean ± SD ($n$ = 2–3).

The concentrations of dHGF in serum beyond 1 or 2 h after single sc and im administrations were sustained at a higher level than those in serum after single and repetitive iv administrations (Figure 1, parts A−C), indicating that dHGF was slowly released from injection sites into the systemic circulation. The concentration−time profiles of dHGF in serum after single sc and im administrations in rats were similar to those of other recombinant proteins such as human erythropoietin (EPO)[17] and human granulocyte colony-stimulating factor (G-CSF).[18] The values of terminal elimination half-life after single sc or im administration were larger than that after iv administration. The prolongation of half-life suggested that the absorption rate may be smaller than the elimination rate (i.e., "flip-flop") as found in G-CSF.[18]

dHGF has been found to ameliorate disorders of liver functions such as hepatic protein synthesis and cholesterol metabolism in various liver-injured rat models[19] and exert preventive effects against various liver injuries.[20] The prolongation of half-life in sc or im administration may be effective in enhancing the pharmacological effects of dHGF as found in EPO and G-CSF.[17,21]

We therefore compared the pharmacological effects of dHGF among iv, sc, and im administration. Serum levels of total protein, albumin, HDL-cholesterol, and liver weight were significantly increased by repetitive iv administration (twice daily for 4 days) of dHGF (0.25 mg/kg), but not by repetitive sc or im administration. However, repetitive sc or im administration of dHGF at 2.5 mg/kg significantly increased these parameters, indicating that repetitive sc or im administration was less effective in exerting the pharmacological effects of dHGF than repetitive iv administration. Although there was no difference in serum level and $AUC_{last}$ value of dHGF between single and repetitive iv administrations (Figure 1A, Table 1), the serum level and the $AUC_{last}$ values after sc or im administration of dHGF were dramatically decreased by repeating its administration (Figure 1, parts B and C, Table 1). Accordingly, the reduced pharmacological effects of dHGF in repetitive sc or im administration seemed to be due to the drastic decrease in serum level and $AUC_{last}$ value of dHGF caused by repeating its administration. These findings suggested that a release of dHGF from injection sites into the systemic circulation declined. One of the mechanisms by which such a phenomenon occurs in repetitive sc or im administration may be due to altered absorption kinetics (related to dermal vascular changes, or other local and/or systemic processes). Such a phenomenon is also observed when recombinant human interlukin-3 (rhIL-3) has been administered by repetitive sc administration once daily for 4 days.[22] However, the decrease of $AUC_{last}$ values of dHGF after repetitive sc administration was much greater than that of rhIL-3. The distinction between rhIL-3 and dHGF may be due to differences in the number of times of repetitive administration and/or in their physicochemical properties.

We next examined distribution of dHGF into liver after iv administration. A large amount of dHGF (53.6% of the injected dHGF) was distributed rapidly (5 min after iv injection) into liver. The high distribution of dHGF into liver may be due to its binding to extracellular matrix components such as heparan sulfate, because dHGF has a high affinity for heparin.[6] It has also been shown that HGF has a high affinity for heparin[2,23,24] and binds to heparin-like substances on the cell surface [25,26] and the extracellular matrix[27] in liver.

dHGF exerts maximal mitogenic activity for adult rat hepatocytes in a dose range of 10 to 500 ng/mL in vitro,[7] suggesting that a hepatic dHGF concentration of 10 ng/g of liver would be sufficient to exert a physiological function. The iv bolus injection of dHGF (0.5 mg/kg) sustained the effective hepatic level for more than 4 h (18.1 ± 4.9 ng/g) (Figure 3A). In addition, when dHGF (0.5 mg/kg/3 h) was administered by infusion, the effective level of dHGF in liver was sustained beyond the steady-state (for more than 6 h) (Figure 3B), suggesting that infusion would be more effective than iv bolus injection in enhancing the pharmacological effects of dHGF.

In conclusion, we demonstrated that dHGF in serum decreased more rapidly after iv injection than after sc and im injections, but that iv administration exerts the most potent pharmacological effect of dHGF among three administration routes. Further, we found that dHGF is distributed mainly and rapidly into liver and is sustained at a higher level in liver than in plasma.

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