Nonlinear pharmacokinetics of hepatocyte growth factor and its control using heparin and protamine

肝細胞増殖因子の非線形体内動態とヘパリンならびにプロタミンによる動態制御

加藤将夫

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CONTENTS

GENERAL INTRODUCTION

Recently, many types of polypeptides such as cytokines or lymphokines have been identified. These compounds induce the differentiation and/or growth of a great variety of cells at very low concentrations in the nM or pM range *in vitro*, and they are undergoing development for application as therapeutic drugs for a wide variety of diseases. In addition, advances in gene recombination techniques allowing the bulk production of such compounds have lead to great expectations about their future use. Although these polypeptides exhibit dramatic biological activity *in vitro*, there are many difficulties to be overcome with regard to their *in vivo* administration. One of the most critical issues is how to deliver such compounds efficiently to target organs and to allow them to display their pharmacological effects most effectively after systemic administration. In general, polypeptides have very short biological half-lives (24,66), which may be one of the reasons they do not always produce a sufficiently powerful effect *in vivo*. For the efficient delivery of polypeptides to the target, a suitable drug delivery system (DDS) must be developed.

Hepatocyte growth factor (HGF) was first identified as a potent mitogen for mature heptocytes. HGF is now widely recognized as a multifunctional cytokine which stimulates DNA synthesis in a variety of types of epithelial and endothelial cells (41,42). HGF is identical to a scatter factor which can stimulate the motility of epithelial cells and also a tumor cytotoxic factor which inhibits the growth of several types of carinoma cell lines (41,42). Since HGF is the most potent

mitogen as far as hepatoctes are concerned and it has been shown to stimulate the repair of the liver, kidney, and lung after injury to these tissues in experimental animals (19,25,51), it is confidently expected that it will be developed as a treatment for certain types of injuries involving the liver or other organs. However, Liu et al. (30) have already demonstrated that the plasma half-life of HGF is very short (~ 4 min). Additionally, a large dose (~ 300 μg/kg body wt) of HGF is usually necessary to obtain a pharmacological effect *in vivo* (19). This could be a stumbling block to developing HGF as a pharmaceutical agent.

The final goal of this study is to discover the reason for such a short plasma half-life. To achieve this, the elimination mechanism of HGF from the circulating plasma needs to be clarified. In addition, based on such a clearance mechanism, we need to construct a drug delivery system (DDS) for HGF which will produce a more potent pharmacological effect *in vivo* at a lower dose. Liu et al. (30) have also reported that the liver is a major clearance organ for HGF under tracer dose conditions. They also proposed that receptor-mediated endocytosis and another unknown uptake mechanism, probably mediated by cell-surface heparan-sulfate proteoglycans (HSPG), in the liver constitute the elimination mechanism for HGF at low doses (30). However, the nonlinear pharmacokinetics of HGF at relatively higher doses has not been examined yet. Furthermore, to develop HGF as a treatment, we must also investigate the clearance mechanism under higher dose conditions where the pharmacological effect can be observed.

With these factors in mind, in this study I have focused on the following three points which reuire clarification:

(i) Characterization of the nonlinear pharmacokinetics of HGF

(ii) Clearance mechanism of HGF over a wide dose range

(iii) Construction of DDS for HGF using heparin, a glycosaccharide which has an affinity for HGF, and protamine, a basic protein which has an affinity for heparin and, possibly, HSPG.

PART I

Heparin-hepatocyte growth factor complex with low plasma clearance and retained hepatocyte proliferating activity

ABSTRACT

Since hepatocyte growth factor (HGF) is known to have affinity for heparin, we studied the binding isotherm and found that HGF has a high-affinity binding site for ^{35}S-heparin with an equilibrium dissociation constant of approximately 0.6 nM. We then analyzed the pharmacokinetic behavior of the heparin-HGF complex in rats. The area under the plasma concentration-time profiles of trichloroacetic acid-precipitable radioactivities from 0 to 30 min after the intravenous administration of the heparin-^{125}I-HGF complex was approximately 3 times that after the administration of ^{125}I-HGF only. Since we previously demonstrated that the liver is the major clearance organ for HGF, the hepatic uptake of ^{125}I-HGF and the heparin-^{125}I-HGF complex was compared. The liver-to-plasma concentration ratio after the intravenous administration of the complex was half that after the administration of ^{125}I-HGF only. Furthermore, the steady-state hepatic extraction ratio of ^{125}I-HGF in perfused rat liver decreased depending on the heparin concentration. In addition, the biological activity of the complex was examined by assessing the ^{125}I-deoxyuridine incorporation in cultured rat hepatocytes. Although the half-effective concentration of HGF increased slightly, namely 2-3 times in the presence of heparin than that in its absence, the maximal activity was not changed. We concluded that the heparin-HGF complex, which retained biological activity, exhibited much lower clearances for hepatic uptake and plasma disappearance than HGF itself. The heparin-HGF complex may thus be utilized as a drug delivery system for HGF.

INTRODUCTION

Hepatocyte growth factor (HGF), with a Mw of 82-85 kD (46,48), has a strong affinity for heparin (10,47,79) and is a most potent mitogen for mature hepatocytes (15). The levels of the HGF activity and HGF mRNA markedly increased both in the plasma and in the liver of rats with hepatitis, which was induced by hepatotoxins such as carbon tetrachloride(CCl_4) or D-galactosamine (2,26,29,50). On the other hand, the HGF receptor on liver cell-surface is down-regulated after a partial hepatectomy or CCl_4-induced hepatitis (15). In CCl_4-intoxicated rats the uptake clearance of HGF in the liver decreased and down regulation of the HGF receptor is suggested to be one of the causes of such a decrease (31). HGF-producing cells are Kupffer, endothelial cells (26,50), and fat-storing (Ito-) cells (42) in the liver; endothelial and mesangial cells (18) in the kidney; and macrophage and endothelial cells (76) in the lungs. Recently, Matsumoto et al. (39) identified "injurin", which induced HGF gene expression.

We previously analyzed pharmacokinetics of HGF in rats using *in vivo* and a perfused liver system and found that HGF was distributed to the liver, adrenal, spleen, kidneys, and lungs after intravenous administration (30). Tajima et al. (67) examined the tissue distribution of the HGF receptor and reported that the HGF receptor distributes much in these organs. The most important organ playing a major role in eliminating HGF from the circulation is the liver (30). On the liver cell-surface there exist HGF receptors with a dissociation constant of 20-40 pM (15) and receptor-mediated endocytosis (RME) of HGF contributes to the hepatic clearance (30). RME plays a key role in clearance not only of HGF, but also of some other biologically important polypeptides including epidermal growth factor; and the concept of "transport

receptor", which acts a transporter and/or clearance mechanism of polypeptides, is also now well established (24,37,61,62,65,66,77,78). HGF is known to bind to heparin-like substance on the cell-surface (81) and extracellular matrix (38) in the liver. We previously showed that HGF is taken up by liver not only via RME, but also via a non-specific mechanism, which probably occurs after the binding to such heparin-like substances (30).

The disappearance of HGF in plasma is very rapid (early phase half life ≈ 4 min, 30). This may be a barrier to develop HGF as a therapeutic drug for certain types of diseases, since large quantities are necessary to show pharmacological effects *in vivo*. Therefore, it is important to construct a drug delivery system which exhibits much longer plasma half-life of HGF. We previously found that when ^{125}I-HGF prebound to heparin was intravenously administered, the plasma disappearance of HGF was delayed to a great extent compared to the administration of ^{125}I-HGF only (31). In the present study we analyzed in details the pharmacokinetics of the heparin-HGF complex in rats using *in vivo* and perfused liver systems. In addition, we investigated the hepatocyte proliferating activity of the complex using primary cultured rat hepatocytes.

RESULTS

In vitro binding study of ^{35}S-heparin to HGF

The binding isotherm between HGF and heparin was analyzed (Fig. 1). The curve linear Scatchard plot indicated the presence of heterogeneous binding site (Fig. 1), and the binding isotherm (Cb vs. Cf) was fitted to the three models; (i) model with a single kind of saturable binding site and a nonsaturable binding, (ii) model with two kinds of saturable binding sites, (iii) model with two kinds of saturable binding sites and a nonsaturable binding. Goodness of the fit was assessed by AIC value, and the data was fitted best to model (iii). The dissociation constants for the binding of heparin to high and low affinity binding sites were 0.57 and 230 nM, respectively.

Plasma disappearance of ^{125}I-HGF or heparin-^{125}I-HGF complex

The effect of heparin on the plasma disappearance of HGF was analyzed (Fig. 2A). The TCA-precipitable radioactivity disappeared rapidly after the intravenous administration of tracer ^{125}I-HGF only, with the area under the plasma concentration time curve from 0 to 30 min ($AUC_{(0\text{-}30)}$) of 11.2 ± 0.0% of dose·min/ml. On the other hand, the plasma concentration time profile was quite different after administration of a mixture of ^{125}I-HGF and heparin with high Mw. The $AUC_{(0\text{-}30)}$ values were 23.6 ± 2.5, 25.7 ± 0.1, and 33.4 ± 0.2% of dose·min/ml after intravenous administration of ^{125}I-HGF with 5, 10, and 20 mg heparin with high MW, respectively. The increase of $AUC_{(0\text{-}30)}$ value by the coadministration of any dose of heparin was statistically significant ($p < 0.05$). The $AUC_{(0\text{-}30)}$ value obtained with 20 mg heparin was thus 3 times that after the administration of ^{125}I-HGF alone. Next, the effect of

heparin with a low MW on the plasma disappearance of HGF was examined. After the intravenous administration of ^{125}I-HGF with 20 mg heparin with a low MW, the $AUC_{(0\text{-}30)}$ value was 25.0 ± 0.0% of dose·min/ml, which was slightly smaller than that after the administration of heparin with a high Mw.

Tissue distribution of ^{125}I-HGF or heparin-^{125}I-HGF complex

The tissue distribution of radioactivity was determined at 10 min after the intravenous administration of a tracer amount of ^{125}I-HGF only or the mixture of heparin and ^{125}I-HGF. After the intravenous administration of ^{125}I-HGF only, the tissue-to-plasma concentration ratio (Kp) in the liver was 3.11 ± 0.15 ml/g of liver and greater than those in other tissues (Fig. 3A). After the intravenous administration of ^{125}I-HGF with heparin, the Kp value was 1.43 ± 0.05 ml/g liver and also greater than those in other tissues (Fig. 3A), but was reduced to 46% of that after the administration of ^{125}I-HGF only. The Kp values in the adrenal, spleen, kidneys, lungs, and duodenum, after the administration of ^{125}I-HGF with heparin, also dropped to 36, 26, 44, 22, and 40% of those after the administration of ^{125}I-HGF alone, respectively (Fig. 3A). When the Kp values were converted to the distribution volume ($V_{d,tissue}$) per kg body wt, the liver value was much greater than those in other tissues (Fig. 3B).

Hepatic handling of heparin-^{125}I-HGF complex in the single-pass perfused rat liver

Since liver is the major clearance organ for HGF (30), the effect of the complex formation with heparin on the hepatic removal of HGF was examined using the single-pass perfused rat liver (Fig. 4). The steady-state hepatic extraction ratio (E_h) of a tracer (0.8 pM) ^{125}I-HGF was 0.51

± 0.01. Sequential perfusions of ^{125}I-HGF with increasing concentrations of heparin (0.1, 0.3, 1, 3 mg/ml) were done, and the E_h value was found to drop sequentially and was 0.34 ± 0.01, 0.10 ± 0.01, 0.08 ± 0.01, and 0.05 ± 0.01, respectively (Fig. 4).

Mitogenic response of primary cultured rat hepatocytes to HGF with different exposure times

The HGF concentration dependence of mitogenic response in cultured rat hepatocytes were examined with different exposure time (Fig. 5). The mitogenic response assessed by ^{125}I-deoxyuridine incorporation after the various exposure time (0.3 - 28 h) with various concentrations of HGF (0 - 250 pM) was determined and found to increase, as the HGF concentration increased, after any exposure time (Fig. 5). In addition, the mitogenic response increased, as the exposure time increased, at any concentration of HGF (Fig. 5).

Mitogenic response to heparin-HGF complex

Mitogenic response to a mixture of HGF (40 pM) and various concentrations of heparin (0 - 3 mg/ml) with high or low Mw were determined using cultured rat hepatocytes (Fig. 6). In the presence of 40 pM HGF only, 50% of the maximum response (obtained at 90 pM HGF) was observed. The mitogenic response to 40 pM HGF was reduced in the presence of a relatively high concentration (> 0.1 mg/ml) of heparin, and 35% or 32% of the maximum response was observed in the presence of 1mg/ml heparin with high or low Mw, respectively (Fig. 6). On the other hand, the effect of heparin on the mitogenic response was minimal in the presence of excess (500 pM) HGF (Fig. 6). Mitogenic response to only heparin was negligible (Fig. 6). The HGF concentration dependence on mitogenic response was examined in the presence or absence of 1

mg/ml heparin (Fig. 7). In addition, the occupations of high and low affinity heparin binding sites on HGF by heparin (1 mg/ml) were estimated based on the binding parameters obtained. It was suggested that most (> 99.9%) of the both binding sites on HGF molecule was occupied by heparin at any concentration of HGF (10 - 500 pM). The half-effective concentration (EC_{50}) was 43.0 ± 1.2 pM in the absence of heparin, and 106 ± 4 or 78.3 ± 2.5 pM in the presence of heparin with a high or low Mw, respectively, while the maximal values of the mitogenic response was comparable in either case (Fig. 7).

DISCUSSION

Since HGF markedly stimulates DNA synthesis of mature hepatocytes (15,42), it is expected to be therapeutic for certain types of liver diseases. However, there are some difficulties before this is realized. That is, the plasma half-life of HGF was very short (30), and large quantities are necessary to show pharmacological effects *in vivo*. Therefore, it is essential to develop drug delivery system (DDS) which increases the plasma residence time of HGF *in vivo* with a minimal change in intrinsic biological activity. Such a necessity for DDS development was also supported by the results of the present study (Fig. 5). Mitogenic response of cultured rat hepatocytes was increased, as the residence time of HGF in the medium increased, at any HGF concentration (Fig. 5). This result suggested the prolongation of the plasma residence time is effective for the increase of biological activity of HGF *in vivo*. The development of DDS for the avoidance of HGF clearance mechanism is thus important to realize the clinical application of HGF.

We designed a complex of heparin and HGF as a DDS of HGF, which is aimed to result in the longer plasma residence time of HGF. HGF has a great affinity for heparin (10,46,79) and binds to heparin-like substances, which exist on cell-surfaces and/or in extracellular matrices (38,81). However, the isotherm of such binding has not been reported yet, so the binding parameters were determined by using an ultrafiltration technique (Fig. 1). Scatchard analysis (Fig. 1) revealed the existence of two kinds of saturable binding with high affinities (Kd values; 0.6, 230 nM).

We previously reported that HGF is taken up by the liver via not only the receptor-mediated endocytosis, but also non-specific uptake mechanism, which is probably related to the adsorption of HGF to

heparin-like substances (30). Therefore, it may be possible that the pharmacokinetic behavior of HGF molecules prebound to heparin is modified. In fact, when ^{125}I-HGF prebound to heparin was administered intravenously, the plasma clearance of ^{125}I-HGF was lower, compared with that after intravenous administration of ^{125}I-HGF only (Fig. 2). In addition, the steady-state hepatic extraction ratio of ^{125}I-HGF, which was measured in a perfused liver system, was decreased to a great extent in the perfusion of heparin-^{125}I-HGF complex than that in the perfusion of ^{125}I-HGF only (Fig. 4). These results suggested that the administration of the heparin-HGF complex was effective to reduce HGF clearance.

The tissue-to-plasma concentration ratio (Kp) of ^{125}I-HGF in several tissues was also examined after the intravenous administration of heparin-^{125}I-HGF complex and found to be less than half of that after intravenous administration of ^{125}I-HGF only in any tissues (Fig. 3A). When the Kp values were converted to the distribution volume ($V_{d,tissue}$) per kg body wt, the value in the liver was much greater than those in other tissues (Fig. 3B). This result suggested that heparin-HGF complex avoids its uptake to the liver and such avoidance of hepatic uptake contributes much to the decrease of HGF plasma clearance. The results that the $V_{d,tissue}$ value of either ^{125}I-HGF or heparin-^{125}I-HGF complex in the liver was much greater than those in other tissues (Fig. 3B) supported our previous finding that the liver is the major organ responsible for the removal of HGF from the circulation (30) and also suggested that this holds true for the heparin-HGF complex.

Although the complex formation of HGF with heparin resulted in the prolongation of HGF retention in plasma, it was possible that heparin blocks the site of the HGF molecule related to the binding to its receptor and inhibits its biological activity. If heparin-HGF complex does not retain the biological activity of HGF, its application for DDS is not

possible. Therefore, we examined its biological activity in cultured rat hepatocytes (Fig. 6). The mitogenic response to HGF (40 pM) has a tendency to decrease as the heparin concentration increased (Fig. 6). When we consider that the distribution volume of heparin is 260 ml/kg body wt (73), the initial heparin concentration in plasma after the intravenous administration of a complex of ^{125}I-HGF and 5 mg/rat heparin, which showed a marked increase in the $AUC_{(0\text{-}30)}$ value compared with ^{125}I-HGF only (Fig. 2A), is estimated to be approximately 0.08 mg/ml. When such a concentration of heparin was used, the mitogenic response was 70 - 80% of that for HGF alone (Fig. 6), indicating that the heparin-HGF complex retained the comparable biological activity of HGF. On the other hand, the mitogenic response to the excess (500 pM) concentration of HGF did not decrease as the heparin concentration increased (Fig. 6). To investigate the mechanism of the slight decrease of HGF activity by heparin, we examined the HGF concentration dependence of DNA synthesis in the presence of 1 mg/ml heparin (Fig. 7). The half-effective concentration (EC_{50}) was approximately 1.8 - 2.5 times larger in the presence of heparin than that in its absence, while the maximal mitogenic effect was comparable both in the presence and absence of heparin (Fig. 7). When we consider that most (> 99.9%) of the heparin binding sites on HGF molecule is occupied by heparin at any concentration of HGF (10 - 500 pM) used, the experimental data shown in Fig. 7 may reflect the cellular handling of the heparin-HGF complex. Considering that EC_{50} in the absence of heparin (43.0 $\pm$ 1.2 pM) was comparable with the dissociation constant of the HGF binding to its receptor (20 - 40 pM), the increase in EC_{50} value by heparin may be due to the decrease in the affinity of HGF to its receptor. To clarify the mechanism in detail, however, we have to obtain more

information, such as a three-dimensional distance between the receptor-binding region and heparin-binding region on HGF molecule.

It is important to demonstrate the biological activity of the Heparin-HGF complex *in vivo* before considering its clinical applications. According to the results of our preliminary experiment using rats administered with α-naphtylisothiocyanate, the intravenous administration of the heparin-HGF complex significantly decreased serum levels of liver enzymes (GOT, GPT, and ALT). The effect of heparin-HGF complex injection was more clearly observed on the T-bilirubin value than the injection of HGF alone (unpublished observation). We are now examining the hepatocyte proliferative activity of the heparin-HGF complex by assessing the incorporation of ^{125}I-deoxyuridine using rats with hepatic malfunctions, and comparing its effect with that of HGF alone.

Although the mechanism of the decrease in HGF distribution to the liver by heparin remains to be resolved, the following possibilities may be considered: (i) Heparin decreased the affinity of HGF to its receptor; (ii) Heparin reduced the HGF binding to heparin-like substances on the liver cell-surface and/or in the extracellular matrix (38,81). As discussed above, the former possibility is likely. The latter possibility is also supported by the fact that heparin decreased the HGF distribution to all the tissue examined (Fig. 3) when we consider that heparin-like proteoglycans are distributed in ubiquitous tissues (59).

One may have doubt as to the usefulness of HGF as a treatment for acute or chronic liver disease, since the level of endogenous HGF concentration in plasma is markedly increased in such a condition, so that the further administration of this growth factor may not be necessary. Ishiki et al (19) however, reported that exogenously administered HGF has a potent antihepatitis effect *in vivo* using mice subjected to 30%

hepatectomy and mice administered with CCl_4 or α-naphtylisothiocyanate. It is still unknown why the further exogenously administered HGF is still active in such a condition. Further study has to be done to clarify this mechanism.

Although HGF is reported to exhibit remarkable proliferative activity in mice with a hepatic malfunction (19), a large dose (1-5 μg/mouse/day) is necessary to show biological activity *in vivo*. Therefore, a drug delivery system for HGF has to be developed for its clinical application. On the other hand, Francavilla et al. (8,9) found that DNA synthesis in liver is remarkably stimulated by a selective portal infusion of a much lower dose (50 ng/kg body wt/day) of HGF after canine Eck's fistula. It is still unknown whether the reason for such a discrepancy in injected dose is the difference in administration route, species differences, and/or discrepancies in experimental systems. To clarify the dependency of proliferative activity of HGF *in vivo* on the injection site and dose, systematic experiments in which the dose, injection site, and injection interval of HGF are variously changed, and the resultant proliferative activities are determined must be performed.

Zarnegar et al. (79) reported that the mitogenic activity of HGF was abolished by 50 μg/ml heparin at low concentrations of HGF. In the present study, the effect of such concentration of heparin on the mitogenic response to HGF was minimal (Fig. 6). The possible reason for this discrepancy may be considered as follows: (i) The Mw and/or source of heparin used in both laboratories are different; (ii) Species differences in amino acid sequence of HGF may impart different receptor binding characteristics; (iii) The difference of cell density in both laboratories may have an effect on the cellular response since the mitogenic response to HGF depends much on cell density (68).

Since heparin has an anti-coagulative activity, we have to consider the possibility of significant side effects when the heparin-HGF complex is administered to liver diseased patients who already exhibit low coagulative profiles. For a clinical application of the heparin-HGF complex in treatment of liver disease, another heparin-like compounds with a high affinity for HGF and a lower anti-coagulative activity (such as heparan sulfate, dextran sulfate) might be used instead of heparin.

Heparin has great affinity also for basic fibroblast growth factor (bFGF, 35,36). Gospodarowicz et al. (11) reported that heparin inhibits the inactivation of bFGF by acid- or heat-treatment. Resengart et al. (57) found heparin to inhibit proteolytic digestion of bFGF by some proteases. However, such a protective effect of heparin on HGF was still unknown.

We conclude as follows: (i) The plasma clearance of HGF was decreased when HGF prebound to heparin and administered; (ii) Such a decrease was mainly due to the decrease in the hepatic uptake of HGF; (iii) Heparin-HGF complex retained the biological activity of HGF. These results suggest that the heparin-HGF complex may be promising to be applied as a novel drug delivery system of HGF. We are now examining its activity to cure of liver disease *in vivo* using hepatotoxin-intoxicated animals.

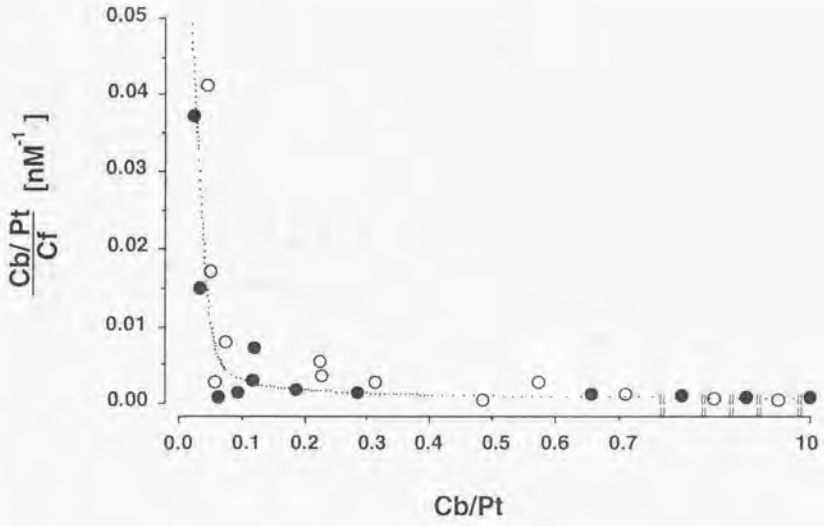


Fig. 1 Scatchard plot of ^{35}S-heparin binding to HGF

^{35}S-heparin (0 - 120 μg/ml) was incubated with 25 nM (○) or 50 nM (●) HGF for 50 min at 25°C and an unbound concentration of ^{35}S-heparin was determined by the ultrafiltration method. Each point represents the mean value of two independent determinations. The broken line is calculated by the non-linear least squares method.

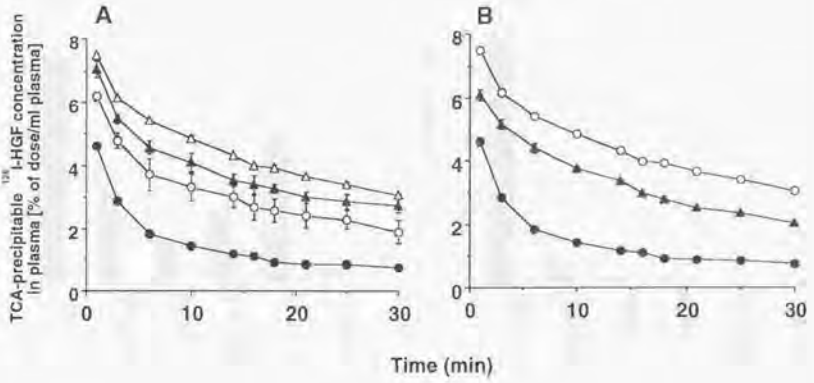


Fig. 2

(A) Time profiles of TCA-precipitable radioactivity after intravenous administration of ^{125}I-HGF only or heparin-^{125}I-HGF complex

After the intravenous administration of only ^{125}I-HGF (●) or a mixture of ^{125}I-HGF and 5 (○), 10 (▲), or 20 (△) mg/rat heparin (Mw 18,000-23,000), TCA-precipitable radioactivities in plasma were measured. Each point and vertical bar represents the mean ± SE of 3 animals.

(B) The effect of the molecular size of heparin on the time profiles of ^{125}I-HGF concentration in plasma.

After the intravenous administration of ^{125}I-HGF only (●) or a mixture of ^{125}I-HGF and 20 mg/rat heparin with a Mw of 18,000-23,000 (○) or 4,000-6,000 (▲), TCA-precipitable radioactivities in plasma were measured. Each point and vertical bar represents the mean ± SE of 3 animals. The vertical bar is not shown when the SE value is smaller than the symbol.

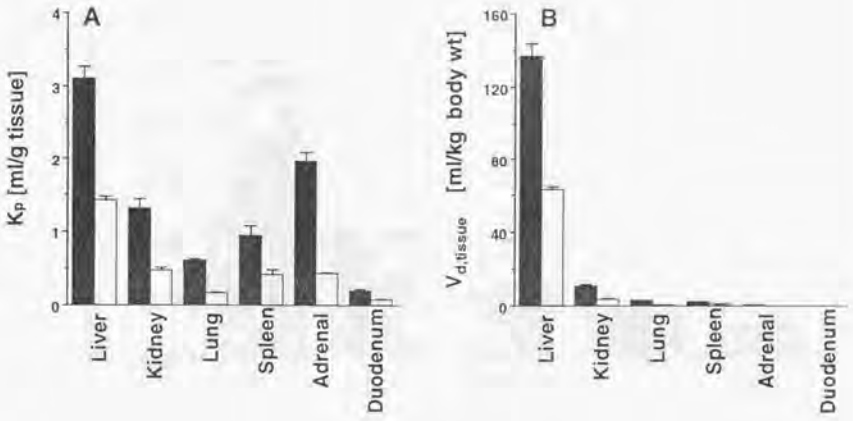


Fig. 3 Tissue-to-plasma concentration ratio (K_p, panel A) and distribution volume ($V_{d,tissue}$, panel B) of radioactivity after the intravenous administration of ^{125}I-HGF only or heparin-^{125}I-HGF complex

Ten min after intravenous administration of only ^{125}I-HGF (closed bar) or a mixture of ^{125}I-HGF and heparin (Mw 18,000-23,000, 20 mg/rat, hatched bar), rats were sacrificed and the liver, adrenal, spleen, kidney, lung, and duodenum were excised. (A) The radioactivities per g tissue were counted and normalized by the TCA-precipitable radioactivity per ml plasma. (B) The distribution volume of ^{125}I-HGF per kg body weight was obtained by multiplying the Kp values in panel A by the tissue weight (g tissue/kg body weight). Each value was the mean ± SE of 3 animals.

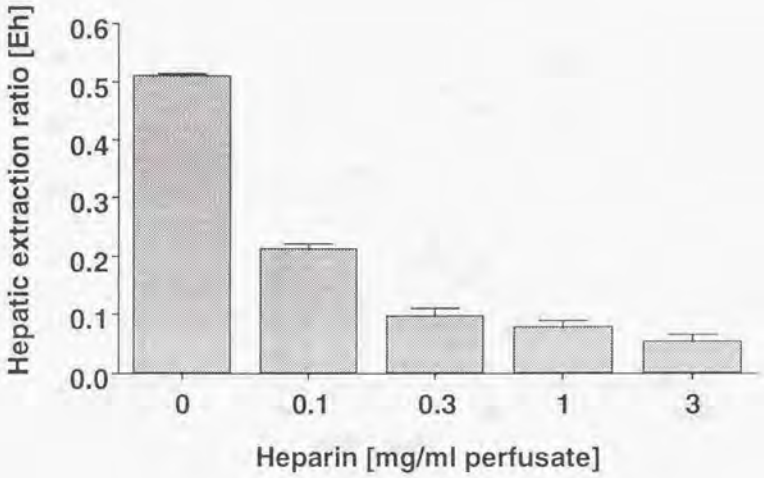


Fig. 4 Steady-state hepatic extraction ratio of ^{125}I-HGF or heparin-^{125}I-HGF complex

Sequential single-pass liver perfusions of a perfusate containing ^{125}I-HGF (0.8 pM) and heparin (0 - 3 mg/ml) were done and the steady-state extraction ratios were determined for each concentration of heparin as described in text. Each value was the mean ± SE of 3 animals.

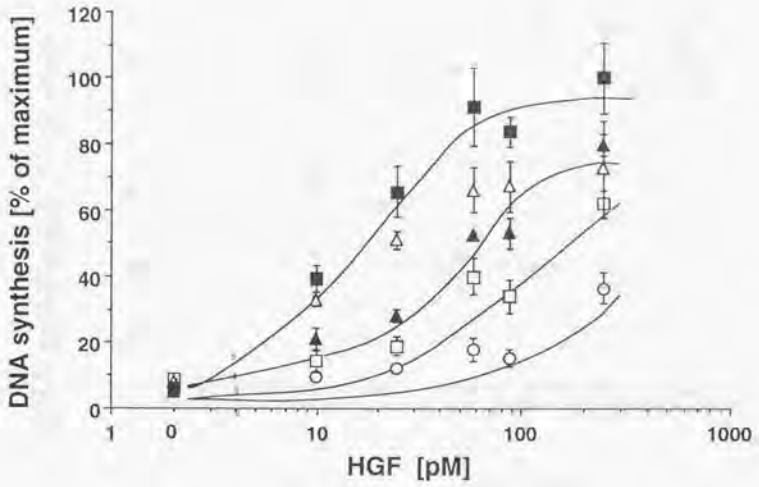


Fig. 5 HGF concentration dependence of mitogenic response in cultured rat hepatocytes with different exposure time

HGF (0 - 250 pM) was added to primary cultured rat hepatocytes. After 0.3 (○), 2 (□), 6 (▲), 10 (△), 16 (■), and 28 (□) h cells were washed and further incubated in HGF-free medium. Twenty two hours after the HGF addition ^{125}I-deoxyuridine was added and its incorporation for 6 h was assayed. Data are expressed as the values normalized by the maximal value (2.63×10^5 cpm/mg protein) of mitogenic response obtained at 28 h in the presence of 90 pM HGF. Each point and vertical bar represents the mean ± SD of three determinations.

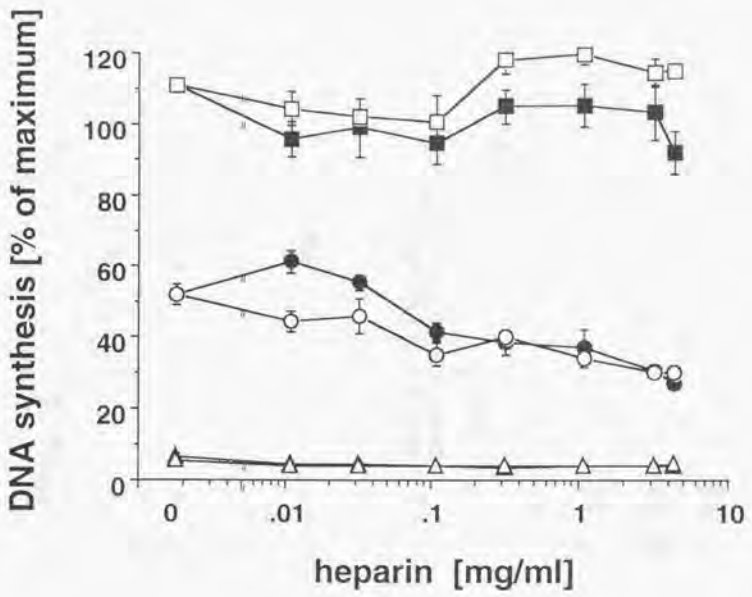


Fig. 6 Mitogenic response of cultured rat hepatocytes to heparin-HGF complex

A mixture of 0 (●,▲), 40 (○,△) or 500 (□,■) pM HGF and indicated concentrations of heparin with a Mw of 18,000-23,000 (●,○,□) or 4,000-6,000 (▲,△,■) was applied to primary culture rat hepatocytes and ^{125}I-deoxyuridine incorporation was measured as described in text. Data are expressed as the values normalized by the maximal value (0.618×10^5 cpm/mg protein) of mitogenic response obtained in the presence of 90 pM HGF alone. Each point and vertical bar represents the mean ± SD of three determinations.

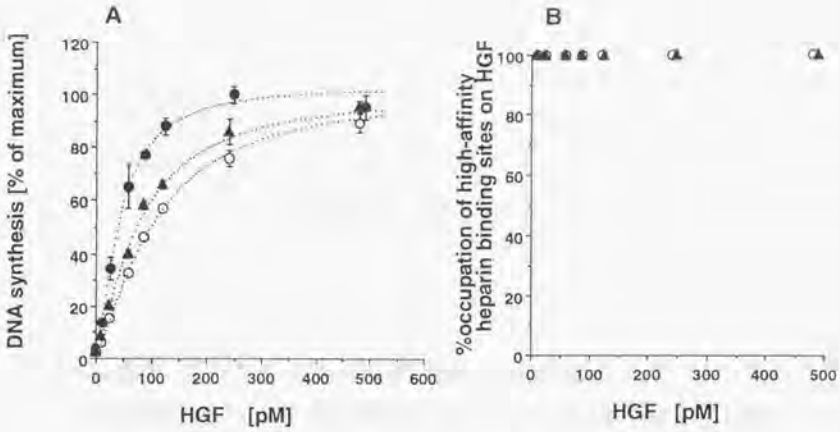


Fig. 7 HGF concentration dependence of mitogenic response in the presence of heparin

Primary cultured rat hepatocytes were incubated with the indicated concentration of HGF in the presence of heparin (1 mg/ml) with a Mw of 18,000-23,000 (○) or 4,000-6,000 (▲) or in their absence (●), and ^{125}I-deoxyuridine incorporation was measured as described in text. Data are expressed as the values normalized by the maximal value (0.618×10^5 cpm/mg protein) of mitogenic response in the presence of 90 pM HGF alone. Each point and vertical bar represents the mean ± SD of three determinations.

PART II

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

ABSTRACT

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. Considering 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, 1/5 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 ^{35}S-heparin to HGF was found showing that one HGF molecule binds to the penta- or hexasaccharide unit. Since 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 while its effect on RME is relatively minor.

INTRODUCTION

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 (41,42). The biological effect of HGF is not restricted to its mitogenic activity, but HGF is identical to the scatter factor which acts as a motogen stimulating the migration of epithelial cells (75). HGF is also a morphogen for epithelial cells and it induces a multicellular architecture (44) as well as being a potent angiogenic factor capable of inducing endothelial cells to proliferate and migrate (6). The biological activity of HGF is exerted through its binding to a specific receptor. The HGF receptor is a protooncogene c-met product (4,14,58) and is expressed on ubiquitous epithelial cells (53,67). 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 (3,71). HGF has an affinity for heparin and can bind to the so-called heparin-like substance on the cell surface and/or extracellular matrix (38,81). Lyon et al. (36) 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 such a heparin-like substance. Both a heparin-binding domain and a receptor-binding domain on the HGF molecule have been identified at the N-terminal half of the α-chain. The former is located on the N-terminal hairpin loop and the second Kringle domain while the latter is within the region of the hairpin loop and the first Kringle domain (43).

HGF also exhibits biological activity *in vivo* in several types of animal with experimentally-induced liver and kidney disease (19,25,56). 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 (8,27) where 0.61-3.05 pmol/kg HGF stimulates hepatocyte growth. One of the reasons why such a high dose is needed may be the short plasma half-life of HGF (~ 4 min)(30). 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 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 (30-33). However, its nonlinear pharmacokinetic behaviour 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 (21,24). Since 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 (21). A mitogenic response by hepatocytes to HGF can be observed in the presence of heparin (45,79,82), suggesting that HGF can bind to its receptor even when it forms a complex with heparin. We previously reported the reduction in HGF clearance following its coinjection with heparin in rats (21). In that study, however, since trichloroacetic acid (TCA)-precipitation was used to determine the plasma concentration of HGF, the experiment was carried out within a short time (< 30 min) of its administration (21). In this study we used enzyme-immuno assay (EIA) to investigate the plasma concentration-time profile of HGF for a long period (~ 48 hour) to examine the effect of heparin on HGF disposition.

RESULTS

Nonlinear elimination of HGF

After intravenous administration of various doses of HGF (1.22 pmol/kg - 12.2 nmol/kg) as bolus injections, plasma concentration-time profiles were investigated using EIA (Fig. 8). At HGF doses below 12.2 pmol/kg, HGF in plasma rapidly disappeared while at more than 12.2 pmol/kg, such plasma disappearance was delayed (Fig. 8). From the data shown in Fig. 8, the CL_{plasma} was obtained (Fig. 9). The CL_{plasma} exhibited a dose-dependent reduction with increasing dose (Fig. 9). This reduction was biphasic, showing both a high-affinity component saturated at relatively low doses (12.2 - 36.6 pmol/kg) and a low-affinity component saturated at relatively higher doses (3.66 - 12.2 nmol/kg) (Fig. 9).

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 following ligation of both the portal vein and hepatic artery (Fig. 10). The plasma disappearance of HGF at all doses examined was delayed in ligated rats compared with the control (sham-operated) rats (Fig. 10). The CL_{plasma} in ligated rats was 14.5±1.1, 9.18±0.90, 0.903±0.349, and 0.538±0.362 ml/min/kg (mean±SE, N=3) at HGF doses of 1.22, 3.66, 36.6 pmol/kg, and 1.10 nmol/kg, respectively, also showing a dose-dependent reduction.

Effect of heparin on the nonlinear elimination of HGF

HGF was first mixed with heparin (0-20 mg/kg) and then given intravenously (Fig. 11A) at an HGF dose of 1.46 nmol/kg when the high affinity component of CL_{plasma} was almost completely saturated (Fig. 9). 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. 11). At more than 0.02 mg/kg heparin, the plasma disappearance of HGF was delayed after injection of heparin-HGF mixture (Fig. 11), compared with that after injection of HGF alone. This effect was heparin-dose dependent (Fig. 11) and reached a maximum when the dose of heparin was 0.4 mg/kg (Fig. 11). Based on the data shown in Fig. 11A, a kinetic parameter, the $AUC_{(0\text{-}180)}$, representing the exposure of HGF in plasma was determined (Fig. 11B). The $AUC_{(0\text{-}180)}$ was 0.0423±0.0039 nmol•min/ml after injection of HGF (1.46 nmol/kg) alone and increased about 21-fold (0.899±0.079 nmol•min/ml) after injection of a mixture with 0.4 mg/kg heparin (Fig. 11B).

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 hour) to accurately estimate the CL_{plasma} (Fig. 12). At an HGF dose of 3.66 pmol/kg near the linear dose range for CL_{plasma} (Fig. 9), HGF was first mixed with enough heparin (0.4 mg/kg), and the mixture was then given intravenously (Fig. 12A). The plasma disappearance of HGF was also delayed, compared with that after an injection of 3.66 pmol/kg HGF alone (Fig. 12A). However, the reduction in CL_{plasma} at an HGF dose of 3.66 pmol/kg was not so marked (Fig. 12A, Table 2), compared with the HGF dose of 1.46 nmol/kg (Fig. 12B, Table 1): At an HGF dose of 3.66 pmol/kg, the 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, the CL_{plasma} of HGF with heparin was only 2.4% that after administration of HGF alone (Table 1). The saturable component of the CL_{plasma} was estimated by subtracting the 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 approximately 10 ml/min/kg for the heparin-HGF mixture, approximately 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. 13). For both ^{35}S-heparins with molecular weights of 21-23 kDa and 12-13 kDa, saturable and nonsaturable components could be observed for the binding with HGF (Fig. 13). The K_d values were 0.310±0.130, 0.487±0.292 nM, the n values were 0.0862±0.0126, 0.121±0.024 mol of heparin/mol of HGF, and the α values were 0.0115±0.0012, 0.0165±0.0018 (mean±calculated SD) for the 21-23 kDa and 12-13 kDa ^{35}S-heparins, respectively (Fig. 13).

DISCUSSION

We have investigated the elimination mechanism of HGF and come to several conclusions: The major clearance organ for HGF is the liver (30), 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 (30,33). Since the nonlinear elimination profile of HGF from the circulation has never been reported previously, we analyzed the plasma concentration-time profiles of HGF after intravenous administration of several different doses (Fig. 8, 10). The present study supports that liver is 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. 10).

Considering that RME contributes to HGF clearance (30,31,33), it may be that its plasma elimination exhibits nonlinearity because of saturated receptor binding and/or subsequent endocytosis. Actually, the CL_{plasma} showed biphasic saturation with increasing HGF doses (Fig. 9); e.g., the CL_{plasma} was reduced at 12.2-36.6 pmol/kg and 3.66-12.2 nmol/kg (Fig. 9). 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 where the plasma concentration ranged from 10 to 100 pM (Fig. 8). Considering that the equilibrium dissociation constant of the HGF receptor is 20-40 pM (15), 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, the CL_{plasma} also exhibited saturation over the dose range 3.66-12.2 nmol/kg (Fig. 9). 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, the 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 (10,47,79). 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. 13). When we gave intravenous HGF (1.46 nmol/kg) prebound to sufficient heparin (0.4 mg/kg), the CL_{plasma} was almost completely reduced to zero, compared with that after the injection of HGF alone (Fig. 12B, 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 (30). 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, the

CL_{plasma} was not completely reduced, but was approximately 20% of that after administration of HGF (3.66 pmol/kg) alone (Fig. 12A, 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 the CL_{plasma} of HGF could still be observed (Table 1). In addition, we and others have reported that the mitogenic response to HGF can be observed even in the presence of heparin in primary cultured rat hepatocytes (21,45,79,82). 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 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 half that of HGF alone. On the other hand, the half-effective concentration of the mitogenic effect of HGF in cultured rat hepatocytes increased 2- to 3-fold following the addition of heparin (21), suggesting that the affinity of heparin-HGF complex for the HGF receptor is also 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. (45) 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 (19,25,56). However, a large dose (>1.22 nmol/kg) is needed to obtain any effect *in vivo* (19,25) although biological activity can be observed at very low (~ 100 pM) concentrations *in vitro* (15,79). 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 (21). In this study the CL_{plasma} of HGF can be reduced to 2% 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. 11), corresponding to 74 units/kg. Considering that an intravenous clinical dose of heparin is usually 100 units/kg (12), this dose of heparin is within the clinical dose range. Therefore, our present study suggests that clinical doses of heparin can almost completely block HGF clearance.

The CL_{plasma} also showed nonlinearity in rats with ligated portal vein and hepatic artery (Fig. 10). This saturation in CL_{plasma} was almost complete and observed at a dose less than 1.22 nmol/kg (15 ml/min/kg at 1.22 pmol/kg and 0.5 ml/min/kg 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 (53,67). 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 21-23 kDa ^{35}S-heparin (Fig. 13). This means that one heparin molecule can bind to approximately 12 HGF molecules and one unit in the heparin molecule which can bind to one 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 12-13 kDa ^{35}S-heparin (Fig. 13). 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. demonstrated that the minimum size binding component in heparan sulfate derived from the liver is a hexasaccharide (36). Heparan sulfate derived from liver possesses more heparin-like properties than a heparan sulfate species derived from other sources (35). Zioncheck et al. (82) 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 heparan sulfate proteoglycan on the cell surface (55,59,70). 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 (59,70). 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 which 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.

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

Dose of HGF	Dose of Heparin [mg/kg]	CL_{plasma} [ml/min/kg]
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

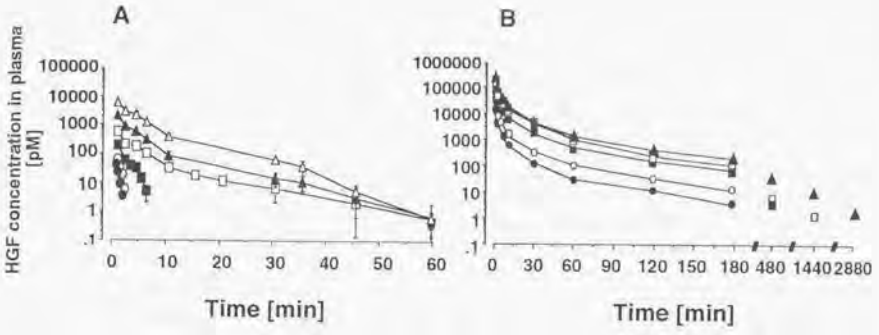


Fig. 8 Plasma concentration-time profiles of HGF after intravenous administration of various doses of HGF

After intravenous administration of 1.22 (●), 3.66 (○), 12.2 (■), 36.6 (□), 122 (▲), or 366 (△) pmol/kg HGF (panel A) and 1.46 (●), 3.66 (○), 6.10 (■), 8.66 (□), or 12.2 (▲) nmol/kg HGF (panel B) as a bolus dose, plasma concentration-time profiles were determined using EIA. Each point and vertical bar represent mean ± SE of 3 rats. The CL_{plasma} of HGF was calculated based on these time profiles and is shown in Fig. 9.

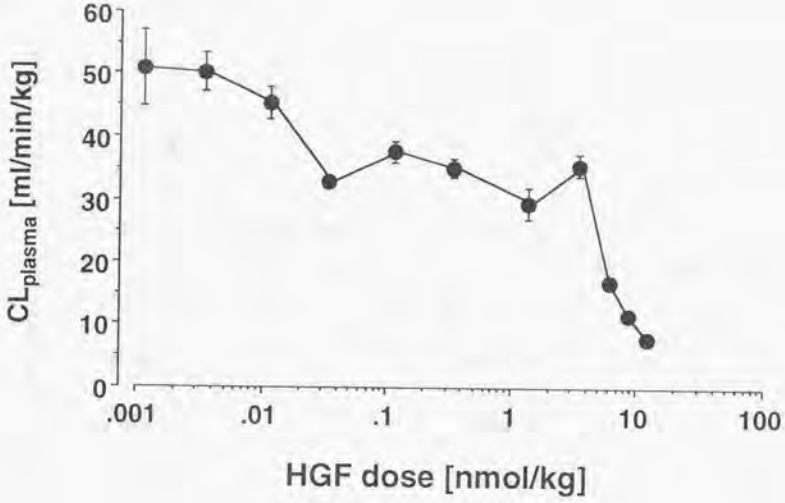


Fig. 9 Two saturable components in the CL_{plasma} of HGF

Based on the plasma concentration-time profiles of HGF shown in Fig. 8, the CL_{plasma} of HGF in normal rats was estimated and plotted against dose. Each point and vertical bar represent mean ± SE of 3 rats. The vertical bar is not shown when the SE value is smaller than the symbol.

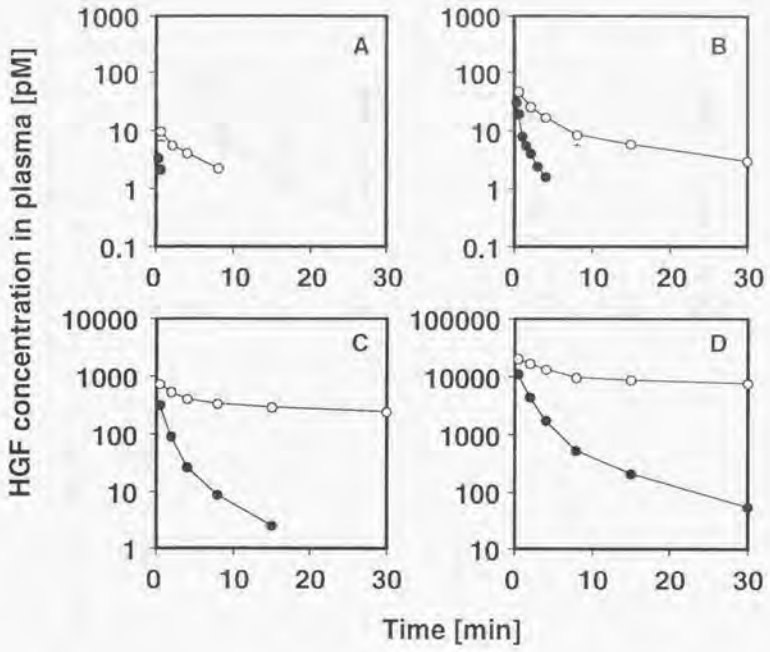


Fig. 10 Elimination profile of HGF in rats with ligated portal vein and hepatic artery

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

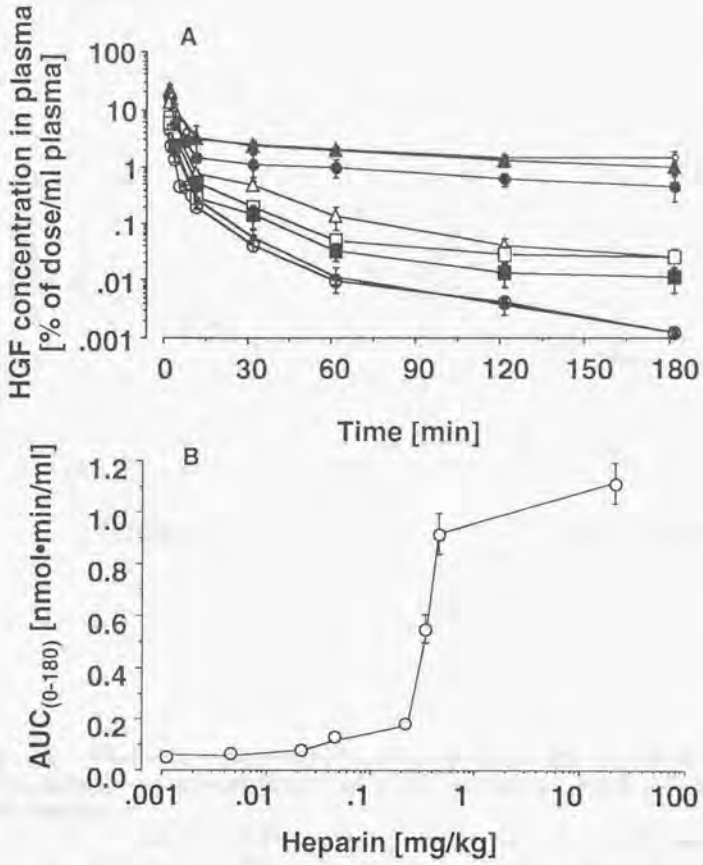


Fig. 11 Plasma concentration-time profiles (A) and $AUC_{(0-180)}$ (B) of HGF after intravenous administration of HGF alone or HGF premixed with heparin

(Panel A) HGF was mixed with heparin to give final heparin doses of 0 (○), 0.004 (■), 0.02 (■), 0.04 (□), 0.2 (△), 0.3 (●), 0.4 (▲), or 20 (○) mg/kg and then injected intravenously. Plasma concentration-time profiles of HGF were determined using EIA and normalized for the injected dose.

(Panel B) Based on the data shown in panel A, the $AUC_{(0-180)}$ was calculated. Each point and vertical bar represent mean ± SE of 3 rats.

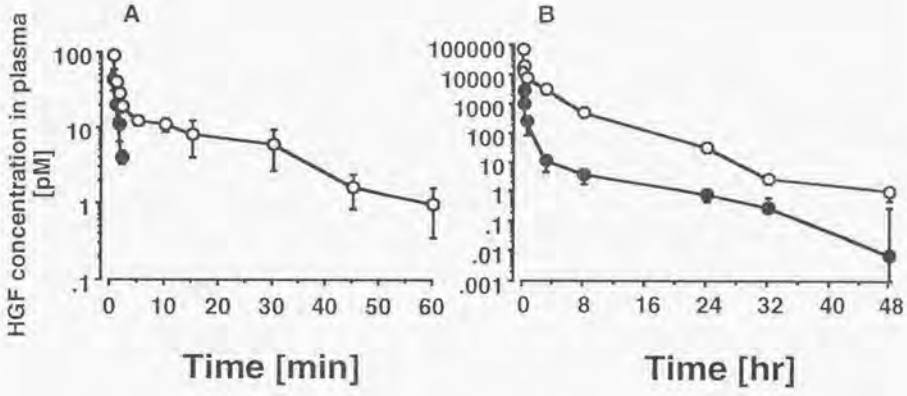


Fig. 12 Plasma concentration-time profiles of HGF after intravenous administration of HGF alone or HGF premixed with heparin

At HGF doses of 3.66 pmol/kg (panel A) and 1.46 nmol/kg (panel B), HGF alone (●) or HGF premixed with sufficient heparin (0.4 mg/kg, ○) was injected intravenously. The plasma concentration-time profiles of HGF were determined by EIA. Each point and vertical bar represent the mean ± SE of 3 rats. The pharmacokinetic parameters obtained are shown in Table 1.

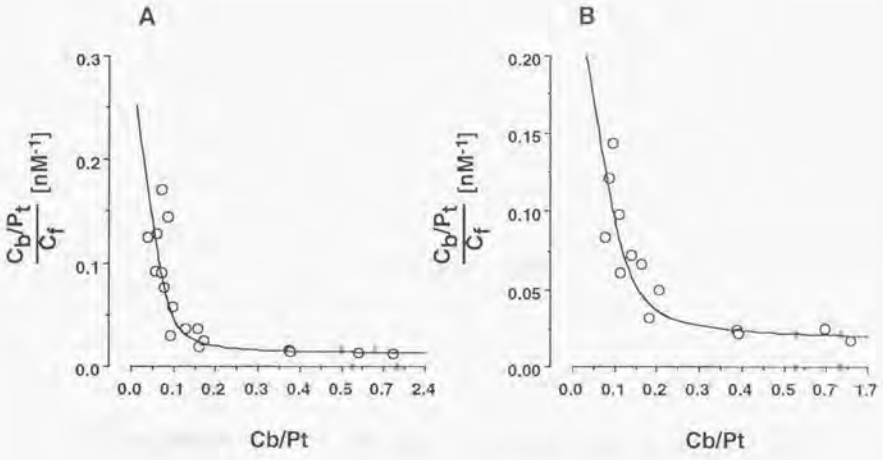


Fig. 13 Scatchard plot representing the binding of ^{35}S-heparin to HGF

The protamine-affinity fraction of ^{35}S-heparin with a molecular weight of 21-23 kDa (panel A) and 12-13 kDa (panel B) was incubated with HGF at 25 °C for 50 min and ^{35}S-heparin binding to HGF was determined by ultrafiltration. The lines in the figure represent the calculated curves obtained by fitting.

PART III

Protamine enhances the proliferative activity of hepatocyte growth factor in rats

ABSTRACT

The effect of protamine on the proliferative activity of hepatocyte growth factor (HGF) was examined in α-naphthylisothiocyanate-intoxicated rats. Protamine pre-injection increased the hepatocyte-labeling index induced by HGF 4-5 fold. A similar effect was also observed in partially hepatectomized rats. Since cell-surface heparin-like substance can bind to HGF, and protamine has an affinity for heparin, protamine may affect HGF pharmacokinetics. In fact, the protamine injection caused a transient increase in plasma HGF concentrations after administration of HGF and, *in vitro*, protamine eluted HGF prebound to heparin-sepharose. Protamine also reduced the plasma clearance of HGF and increased 2.5-fold the exposure of hepatocytes with HGF *in vivo*. The enhancing effect of protamine on the mitogenic response of hepatocytes to HGF was also observed *in vitro* (approximately 2-fold following protamine pretreatment compared with HGF alone), suggesting that the enhancement effect of protamine on HGF-induced liver regeneration results from dual effects exerted by protamine, (i) lowering the overall elimination of HGF and (ii) direct stimulation of hepatocyte mitosis induced by HGF.

INTRODUCTION

Hepatocyte growth factor (HGF) is a heterodimer protein with a molecular weight of 82-85 kD (41,42). HGF stimulates proliferation of a variety types of epithelial cells including hepatocytes (15,18,40,51,79). Its gene expression is increased not only when there is hepatic damage, such as partial hepatectomy (50,69) and carbon tetrachloride poisoning (2,26), but also following renal (17) and pulmonary injury (51). In such cases HGF levels in circulating plasma are increased and, therefore, HGF is believed to be a hepatotrophic, renotrophic, and pulmotrophic factor (17,51,69).

HGF is a basic polypeptide and one of the heparin binding proteins (10,43). HGF can bind to heparan sulfate expressed on the surface of ubiquitous cells and in the extracellular matrix (38,81). Mutational deletion of its N-terminal hairpin loop or second kringle domain reduces the affinity of HGF for heparin, suggesting that these structures are the heparin binding domains on the HGF molecule (43). An oligosaccharide moiety in heparan sulfate required for the binding to HGF has also been identified and is different from that for binding to basic fibroblast growth factor, another heparin binding protein (70). Low concentrations (< 0.1-10 μg/ml) of sulfated oligosaccharides of sufficient length (6 glucose units) induce dimerization of HGF and also increase its mitogenic effect on cultured rat hepatocytes (82). This effect may result from stabilization of the HGF dimer, which stimulates dimerization of the HGF receptor on the cell surface (82).

HGF markedly accelerates regeneration of damaged organs in experimental animals with hepatic and renal failure (19,25). However, a

large dose (> 100 μg/kg) is usually required to exert such a pharmacological effect (19,25). This may be one of the stumbling blocks for the clinical application of HGF. We have trying to identify the way in which it is cleared from the circulation (30-33), and have suggested that both receptor-mediated endocytosis (RME) and the other low affinity uptake system, probably mediated by a cell-surface heparin-like substance in the liver, are involved in the systemic clearance of HGF (30-33). When HGF is premixed with heparin and then given intravenously, its plasma clearance is reduced (21,31). Thus, a heparin-HGF complex like this may be used to increase the plasma residence time of HGF. Such an inhibitory effect of heparin on HGF clearance possibly comes from occupation of the heparin-binding domain on the HGF molecule by heparin which results in a reduction in HGF binding and subsequent internalization through cell-surface heparin-like substance (21,30-33). However, high concentrations (>100 μg/ml) of heparin reduces the mitogenic activity of HGF (21). In addition, heparin has anti-coagulant activity. Therefore, further studies need to be performed to develop a reliable and efficient drug delivery system (DDS) for HGF.

Protamine is a basic protein and also has an affinity for heparin and it has been used clinically to neutralize any excessive pharmacological effect exerted by heparin (1). The molecular weight of protamine is usually around 4 kD, and more than half its amino acid sequence consists of arginine. If protamine can bind to cell-surface heparin-like substance and inhibit the binding of HGF to this substance, it may be that it can be used as another type of DDS to increase the HGF plasma residence time. Hence, in the present study, we examined the effect of protamine on both the proliferative activity and pharmacokinetics of HGF in rats.

RESULTS

Effect of protamine on liver regeneration induced by HGF in rats with liver damage in vivo

Protamine (1.6 mg/kg body wt) was injected intravenously into ANIT-intoxicated rats 10 min prior to administration of HGF (300 μg/kg), and hepatocyte labeling indices were determined at each time after ANIT intoxication (Fig. 14). The labeling indices after the administration of protamine prior to HGF injection were 0.95 ± 0.08, 1.37 ± 0.33, 4.63 ± 1.01, 3.87 ± 0. 45, 5.02 ± 1.08 and 1.32 ± 0.32 % (mean ± S.E, n = 6) 12, 24, 48, 72, 96 and 120 hour after ANIT treatment, respectively (Fig. 14). These values were 1.2, 2.4, 4.9, 3.8, 2.1 and 1.4 times that following the injection of HGF alone, respectively (Fig. 14). On the other hand, when protamine alone was injected, the labeling indices were much lower, compared with those after administration of HGF alone and protamine prior to the injection of HGF, at any time after ANIT intoxication (Fig. 14).

To examine the protamine dose-dependence, a similar experiment with various doses of protamine was performed and the labeling indices were determined 48 hour after ANIT intoxication (Fig. 15A). Protamine alone could not stimulate liver regeneration in ANIT-intoxicated rats (Fig. 15A). The labeling index increased in a protamine dose-dependent manner when the dose of protamine was increased from 0 to 1.6 mg/kg, and the peak value was reached at 1.6 mg/kg protamine (Fig. 15A). When the dose of protamine was further increased to over 1.6 mg/kg, a dose-dependent reduction in labeling index was observed (Fig. 15A). The effect of protamine on liver regeneration induced by HGF fell to

almost the control level when the dose of protamine was 6.4 mg/kg (Fig. 15A). An enhancing effect of protamine on liver regeneration induced by HGF was also found in partially (30%) hepatectomized rats (Fig. 15B). The protamine dose-dependence in the labeling indices was similar to that in ANIT-intoxicated rats (Fig. 15A, 15B). When the dose of protamine was increased, the peak value of the labeling index occurred at a protamine dose of 1.6 mg/kg (Fig. 15B).

Effect of protamine on bilirubin concentration and activity of liver cytosolic enzymes in ANIT-intoxicated rats

To examine whether protamine promotes the repair of liver integrity induced by HGF in ANIT-intoxicated rats, we determined the change in BIL and activity of liver cytosolic enzymes such as GPT, LAP, ALP, and γ-GTP in serum from rats after administration of HGF alone or protamine prior to HGF injection (Fig. 16). Protamine alone did not reduce the BIL or the activity of liver cytosolic enzymes in serum (Fig. 16). The increase in BIL and activity of liver cytosolic enzymes in serum caused by ANIT administration was significantly countered by injection of HGF (300 μg/kg) alone (Fig. 16). When protamine at a dose of 0.8 or 1.6 mg/kg was administered prior to HGF injection, the serum level of γ-GTP was significantly lower than that after injection of HGF alone (Fig. 16). Protamine slightly enhanced the reduction produced by HGF in BIL, GPT, and LAP although this effect was not significant (Fig. 16).

Effect of protamine on HGF clearance from the circulation in ANIT-intoxicated rats

To examine whether protamine reduces the clearance of HGF from the circulation, plasma concentration-time profiles of HGF in ANIT-intoxicated rats were determined after intravenous administration of HGF alone or HGF following protamine treatment (Fig. 17). The elimination of HGF from plasma after injection of HGF following protamine treatment was slower, compared with that after administration of HGF alone (Fig. 17). The AUC after administration of HGF following protamine injection was 2.48-fold that after HGF injected alone (Table 2). CL_{plasma}, V_1 and V_{dss} after administration of HGF following protamine injection fell to 39.5 %, 34.7 %, and 19.1 % that after administration of HGF without protamine treatment, respectively (Table 2).

To examine whether the stimulant effect of protamine on liver regeneration induced by HGF can be attributed to the increase in HGF AUC produced by protamine pre-injection, the hepatocyte labeling index was plotted against AUC (Fig. 18). The labeling index at 300 μg/kg HGF following 1.6 mg/kg protamine treatment was 5.23 ± 0.99 %, much higher than that after administration of HGF alone at dose of 500 μg/kg (0.530 ± 0.104 %) (Fig. 18) although in both cases the AUC had almost the same value (Fig. 18).

Effect of protamine on DNA synthesis rate induced by HGF and EGF in primary cultured rat hepatocytes

To examine the direct effect of protamine on hepatocytes, we examined the effect of protamine on DNA synthesis in primary cultured hepatocytes in the presence of HGF (Fig. 19A, 19B). When the protamine concentration was increased to 12.5 μg/ml, no significant change in the DNA synthesis rate induced by HGF was observed in hepatocytes cultured

for 3 hours and 24 hours (Fig. 19A, 19B). When the protamine concentration in the medium was further increased to 25 μg/ml, the DNA synthesis in hepatocytes cultured for 24 hours was increased approximately 2-fold, compared with that in the presence of HGF alone (Fig. 19B). The DNA synthesis rate in hepatocytes in the presence of any concentration of HGF was inhibited almost completely when the protamine concentration in the medium was 200 μg/ml (Fig. 19A, 19B). To examine whether the enhancing effect of protamine is specific to HGF, we performed the same experiment with EGF (Fig. 19C). In the presence of 6-25 μg/ml protamine, the DNA synthesis was increased approximately 2-3-fold compared with that of EGF alone (Fig. 19C). When the concentration of protamine was increased to 50 and 200 μg/ml, the DNA synthesis of hepatocytes was inhibited (Fig. 19C).

Protamine causes a transient increase in the plasma concentration profile of HGF after intravenous administration of HGF

To support the hypothesis that protamine competes with HGF for binding to heparin-like substance *in vivo*, we studied the effect of protamine injection on the plasma concentration-time profile of HGF in normal rats (Fig. 20). After intravenous administration of HGF (1 μg/kg), plasma HGF concentrations fell rapidly (Fig. 20). After various doses of protamine (0.48-20 mg/kg) were injected, the plasma concentrations of HGF increased immediately in a protamine dose-dependent manner (Fig. 20). However, such a protamine dose-dependence differed from that for the enhancing effect on the labeling index (Fig. 15) and reached a maximum at 20 mg/kg protamine (Fig. 20).

Protamine elutes HGF prebound to heparin-sepharose in a column chromatography experiment.

HGF bound to heparin in a heparin affinity column could not be washed off by PBS, but was easily eluted with 2M sodium chloride (Fig. 21A). The recovery of HGF from the heparin column was 85.7 % (Fig. 21A). To further support the hypothesis of competition for the binding of HGF to heparin by protamine, we added protamine (20 mg/ml) to the heparin affinity column prebound with HGF (Fig. 21B). The HGF bound to the column was eluted by addition of protamine and the recovery of HGF was 84.4 % (Fig. 21B). After elution with protamine, only a small amount of HGF was further eluted by 2M sodium chloride (Fig. 21B). In this analysis we confirmed that the determination of HGF by EIA was not influenced by 20 mg/ml protamine (data not shown).

DISCUSSION

In the present study, we found that protamine enhances HGF-induced liver regeneration when protamine is administered prior to injection of HGF (Fig. 14). Such an enhancing effect of protamine was found both in ANIT-intoxicated rats and partially hepatectomized rats (Fig. 15), and the protamine dose-dependence in the hepatocyte labeling indices was almost identical in both cases (Fig. 15A, 15B), suggesting that this effect may be general for a number of liver diseases. Protamine also significantly reduces γ-GTP further (Fig. 16) at 300 μg/kg HGF while, at 50 μg/kg HGF, BIL and the activity of all cytosolic marker enzymes examined were significantly reduced by preinjection of protamine, compared with those with HGF alone (data not shown). Thus, the effect of protamine is also observed in the repair of liver function. The dosage of protamine in clinical situations is 10-15 mg for the neutralization of 1000 units heparin (52). Since the regular clinical single dose of heparin is 100 units/kg intravenously, 1.0-1.5 mg/kg protamine is usually used as an antidote for heparin. In the present study, we required 1.6 mg/kg protamine to observe its maximum enhancing effect on liver regeneration (Fig. 15). Thus, this dose of protamine is very similar to the clinical dose and, therefore, may be also used in clinical situations. We should also note that the dose of protamine should be strictly controlled since a higher dose of protamine reduces the mitogenic response to HGF (Fig. 15A and Fig. 19) probably because of its cytotoxic effect.

The CL_{plasma} of HGF was reduced by preadministration of protamine (Fig. 17 and Table 2). We consider that the likely mechanism involves inhibition of the nonspecific clearance of HGF by protamine.

HGF has two binding sites on epithelial cell surfaces, one is the HGF receptor, a specific binding site, and the other is heparin-like substance, which has a lower affinity for HGF (15,42). In our previous study, we suggested that one of the major clearance mechanisms for HGF is its nonspecific uptake in the liver probably mediated by heparin-like substance (30-33). Considering that protamine has a high affinity for heparin (1) and can elute HGF molecules prebound to heparin-sepharose (Fig. 21), a transient increase in plasma HGF after intravenous administration of protamine (Fig. 20) may reflect the transfer of HGF molecules bound to the heparin-like substance on cell surfaces and/or extracellular matrix of various tissues into the circulating plasma following injection of protamine. Thus, protamine and HGF bind to the same region of the heparin-like substance or, at least, to a similar location so that each compound can affect the binding of the other.

There are two possible mechanisms for the enhancing effect of protamine on HGF-induced liver regeneration *in vivo*: one is the increase in HGF AUC which results from inhibition of the nonspecific uptake of HGF by protamine (Fig. 17), the other is a direct stimulatory effect on the mitogenic response of hepatocytes to HGF (Fig. 19B). Protamine increases HGF AUC 2.5-fold (Table 2) while the increase in the liver regeneration, assessed as the area under the time-course of the labeling index after ANIT-intoxication, was approximately 5-fold (Fig. 14). Therefore, the enhancing effect of protamine on HGF-induced liver regeneration can be partially explained by increasing the exposure of hepatocytes to HGF. As shown in Fig. 17, protamine increases the AUC of HGF. However, even after administration of 500 μg/kg HGF the AUC was almost identical to that after administration of 300 μg/kg HGF

following protamine treatment, the labeling index in the former case being only 1/10 that of the latter (Fig. 18). This result suggests that the enhancing effect of protamine on HGF-induced liver regeneration cannot be explained simply by increasing the plasma concentration of HGF. Thus, the mechanism of the effect of protamine is not principally related to its inhibitory effect on HGF clearance. The difference in the protamine dose-dependence between the labeling index (Fig. 15) and plasma disappearance of HGF (Fig. 20), where a maximum effect can be observed at 1.6 and 20 mg/kg protamine, respectively, also supports that the enhancement effect of protamine on liver regeneration cannot be fully explained by such an indirect effect. In fact, the DNA synthesis in hepatocytes induced by HGF was increased by approximately twice the direct stimulatory effect of protamine (Fig. 19B). Therefore, we consider that the effect of protamine on the labeling index *in vivo* can be explained by also considering such a direct effect on hepatocytes as well as an indirect effect on HGF clearance.

The effect of protamine on several cytokine receptors has been investigated (16,34,60). Lokeshwar et al. (34) reported that protamine induced an increase in the number of epidermal growth factor (EGF) receptors by activating cryptic or inactive receptors to become functionally active in Swiss 3T3 cells and human epidermoid carcinoma A431. Protamine also increases EGF-induced phosphorylation of the EGF receptor. In the present study, we also found that protamine enhanced EGF-induced DNA synthesis in hepatocytes *in vitro* (Fig. 18C). This indicates that the direct enhancement effect of protamine on hepatocyte DNA synthesis is not specific to HGF. Sacks and McDonald (60) have also reported that protamine enhanced the insulin-induced

autophosphorylation activity of insulin receptors. Like EGF and insulin receptors, HGF receptors are also transmembrane protein tyrosine kinase (PTK) receptors (3). The diverse biological actions of HGF are a result of signaling through this receptor (4,14,58). According to current thinking, HGF activates its corresponding PTK receptors by inducing receptor-dimerization and autophosphorylation as a first step in an intracellular signaling cascade (13). Therefore, such an interaction of protamine with the HGF receptor or its signal transduction cascade may occur, resulting in the increase in DNA synthesis.

The suppressive effect of protamine on BIL and the activity of cytosolic enzymes, except γ-GTP, was not marked or non-existent (Fig. 16). Since the suppressive effect of HGF alone on BIL and the activity of cytosolic enzymes could not be further increased even when the dose of HGF was raised to 710-1000 μg/kg (data not shown), we consider that 300 μg/kg HGF exerts an almost maximum effect in suppressing BIL and the activity of these cytosolic enzymes in serum in ANIT-intoxicated rats.

We conclude that protamine enhances HGF-induced liver regeneration *in vivo*. Such an effect of protamine can be explained by its dual effects, (i) a direct stimulatory effect on hepatocyte DNA synthesis and (ii) indirect effect on HGF clearance which results in an increased exposure to HGF.

Table 2. Comparison of the pharmacokinetic parameters of HGF in ANIT-intoxicated rats after intravenous administration of HGF alone and protamine prior to the injection of HGF

	AUC [μg•min/ml]	$CL_{(plasma)}$ [ml/min/kg]	V_1 [ml/kg]	Vd_{ss} [ml/kg]	MRT [min]
HGF alone (300mg/kg)	9.98 ± 0.28	32.1 ± 0.9	131 ± 4	$1.10 \times 10^{3} \pm 0.17 \times 10^{3}$	$1.08 \times 10^{3} \pm 0.11 \times 10^{3}$
+Protamine (1.6mg/kg)	24.7 ± 3.2	12.7 ± 1.4	45.3 ± 6.5	389 ± 72	$2.36 \times 10^{3} \pm 1.49 \times 10^{3}$

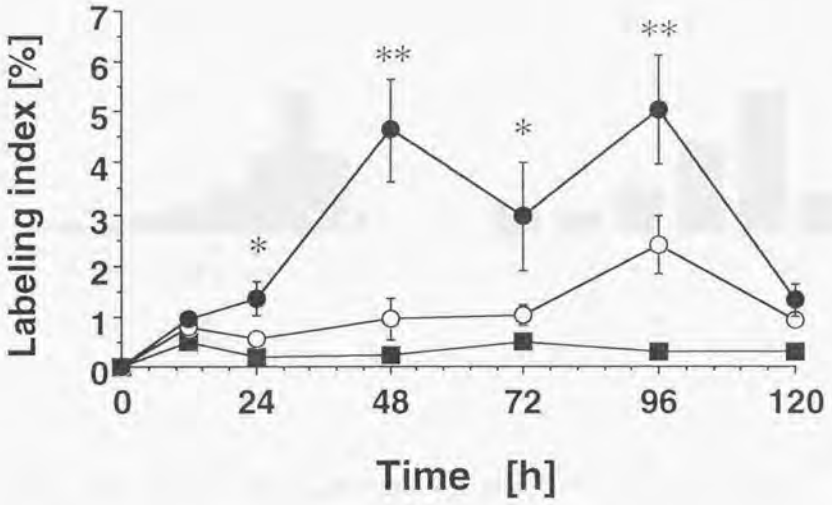


Fig. 14 Time-profiles of DNA synthesis in hepatocytes of ANIT-intoxicated rats treated with HGF alone or protamine prior to HGF injection

ANIT-intoxicated rats were treated with HGF (300 μg/kg) alone (○), protamine (1.6 mg/kg) prior to injection of HGF (●) or protamine alone (■) and the labeling index in hepatocytes was determined at the designated times after ANIT intoxication. Each value and vertical bar represent the mean ± SE of 3-6 rats. *Significantly different from HGF alone ($p < 0.05$); ** ($p < 0.01$).

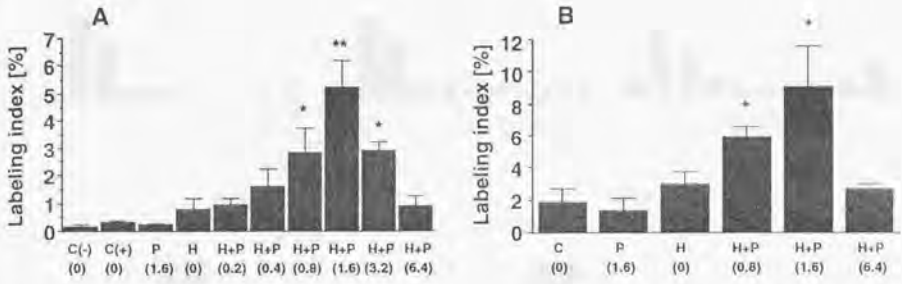


Fig. 15 Effect of protamine on the liver regeneration induced by HGF (A) in ANIT-intoxicated, as well as partially hepatectomized (B), rats

ANIT-intoxicated rats were treated with HGF (300 μg/kg) alone (H), various doses of protamine prior to the injection of HGF (H+P) or protamine (1.6 mg/kg) alone (P), and the labeling index in hepatocytes was determined 48 h after ANIT intoxication (panel A). Similar experiments with HGF were also performed in rats after partial (30%) hepatectomy (panel B). C(+) and C(-) represent ANIT-intoxicated rats treated with saline and non-intoxicated rats treated with saline, respectively. Numbers indicated in parenthesis represent the dose of protamine (mg/kg). Each value and vertical bar represent the mean ± SE of 3-5 rats. *Significantly different from HGF alone ($p < 0.05$); ** ($p < 0.01$).

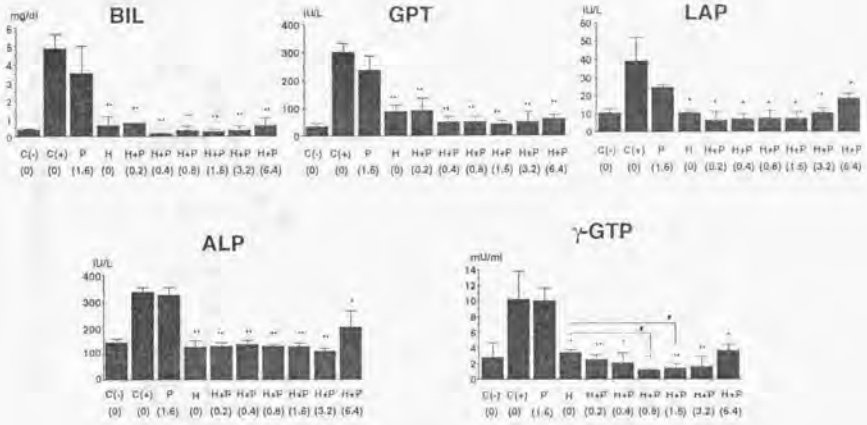


Fig. 16 Change in bilirubin concentration and activity of liver cytosolic enzymes in serum in ANIT-intoxicated rats treated with HGF alone or protamine prior to the injection of HGF

ANIT-intoxicated rats were treated with HGF (300 μg/kg) alone (H), various doses of protamine prior to the injection of HGF (H+P), or protamine (1.6 mg/kg) alone (P). Bilirubin concentration and activity of liver cytosolic enzymes in serum were determined 48 h after ANIT intoxication. C(+) and C(-) represent ANIT-intoxicated rats treated with saline and non-intoxicated rats treated with saline, respectively. Numbers indicated in parenthesis represent the dose of protamine (mg/kg). Each value and vertical bar represent the mean ± SE of 3 rats. *Significantly diff erent from C(+). ($p < 0.05$); ** ($p < 0.01$); #Significantly different from HGF alone ($p < 0.05$).

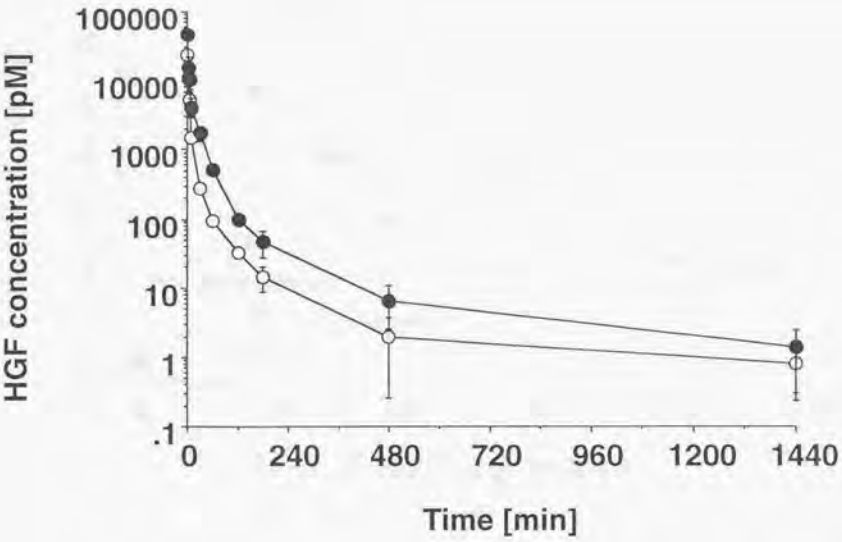


Fig. 17 Effect of protamine on the pharmacokinetics of HGF in ANIT-intoxicated rats

24 h after ANIT-intoxication, HGF (300 μg/kg) alone (○) or protamine (1.6 mg/kg) followed by HGF (300 μg/kg) (●) was given intravenously and plasma HGF concentrations were determined using EIA. The pharmacokinetic parameters obtained are shown in Table 1. Each value and vertical bar represent the mean ± SE of 3 rats.

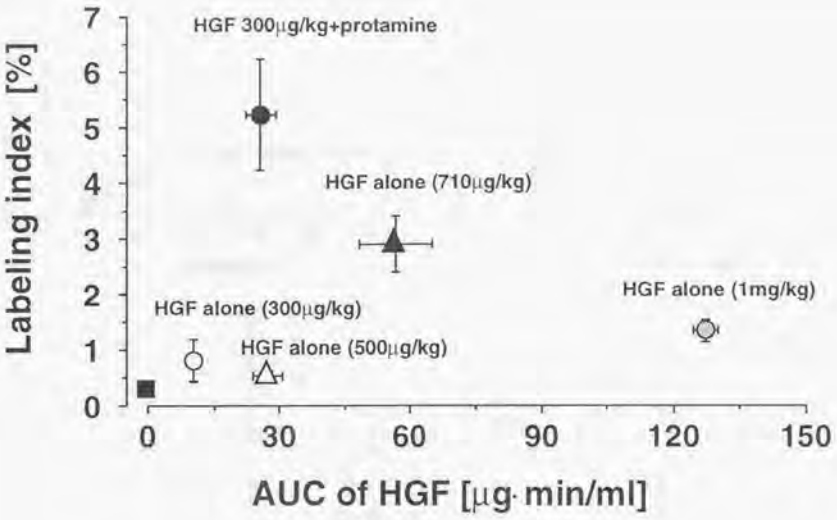


Fig. 18 Relationship between HGF AUC and liver regeneration in ANIT-intoxicated rats

24 h after ANIT intoxication, the indicated doses of HGF (0, 300, 500 μg/kg) alone or protamine (1.6 mg/kg) followed by HGF (300 μg/kg) were given intravenously and plasma HGF AUCs were determined. The ANIT-intoxicated rats were treated with the same doses of HGF alone or protamine prior to the injection of HGF and the labeling index in hepatocytes was thus obtained 48 h after ANIT intoxication. Labeling indices were plotted against AUCs after the corresponding dose. Each value and vertical bar represent mean ± SE of 3-5 rats.

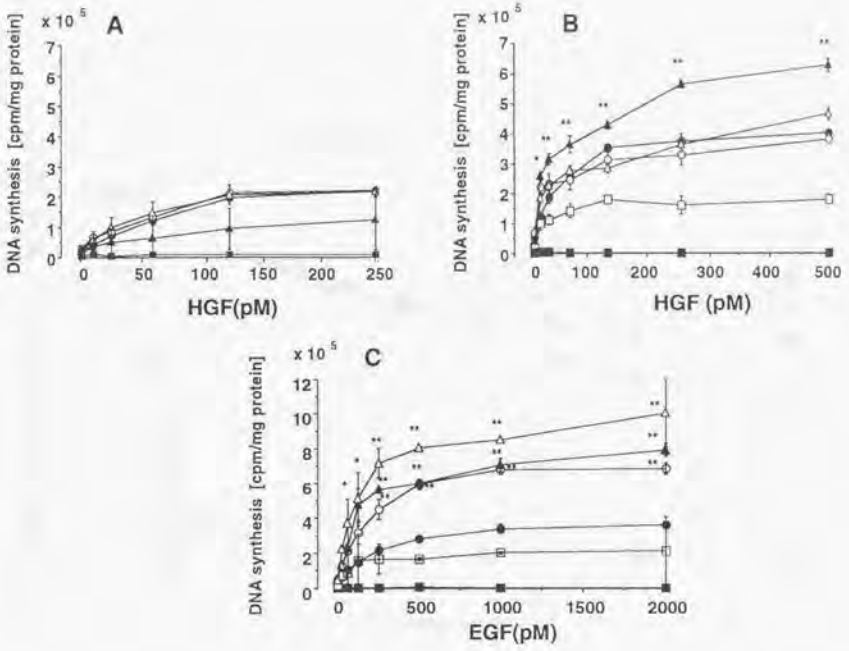


Fig. 19 Effect of protamine on the mitogenic response to HGF and EGF in primary cultured rat hepatocytes

In 3 (panel A) or 24 hours (panel B, C) primary cultured rat hepatocytes, protamine was applied to give final protamine concentrations of 0 (●), 6 (○), 12.5 (△), 25 (▲), and 200 (■) μg/ml. Ten minutes later, HGF was applied to give the indicated final concentrations, followed by the determination of DNA synthesis. Each value and vertical bar represent mean ± SE of 3 rats. * $p < 0.05$, ** $p < 0.01$: Significantly different from protamine concentration of 0.

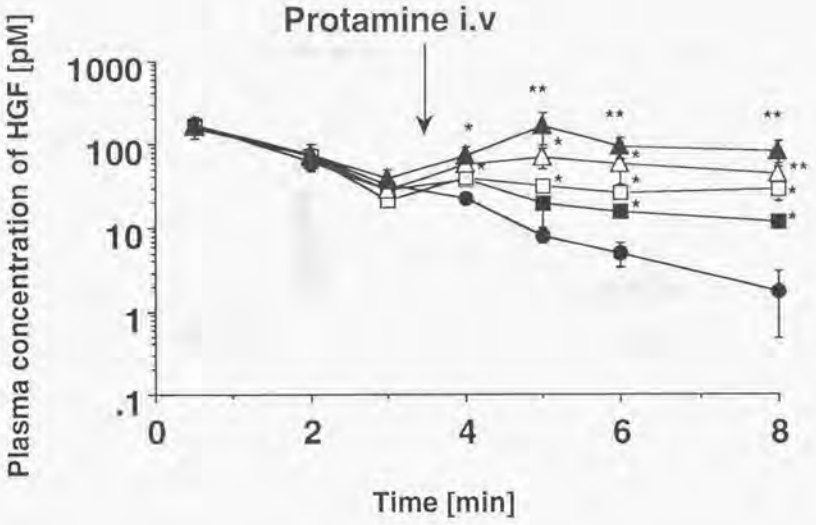


Fig. 20 Effect of protamine injection on the plasma elimination of HGF

1 μg/kg HGF was injected through the penis vein of normal rats. At indicated times, blood was withdrawn through the left external jugular vein. At 3.5 min, 250 μl saline in control rats (●) or protamine at 0.48 (■), 1.6 (□), 5.0 (△), or 20 mg/kg (▲) dissolved in 250 μl saline was injected through the penis vein and blood samples were collected. The plasma concentrations of HGF were determined by EIA. Each value and vertical bar represent mean ± SE of 3 rats. *Significantly different from control ($p < 0.05$); ** ($p < 0.01$).

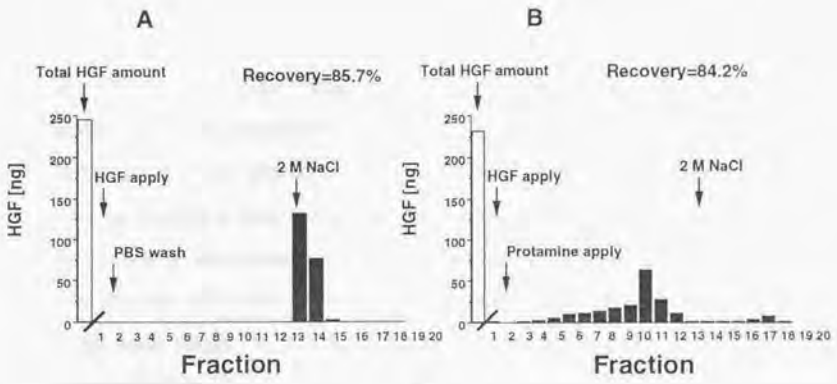


Fig. 21 Protamine elutes HGF prebound to a heparin-immobilized column

1 ml HGF (250 ng) dissolved in PBS was applied to a heparin-immobilized column (1 ml bed volume). The column was then eluted with PBS (A) or protamine (20 mg/ml) (B) and subsequently 2 M sodium chloride. The amount of HGF in the eluate(1 ml / each fraction) was determined by EIA.

CONCLUSION AND FUTURE ASPECTS

In this study the nonlinear pharmacokinetics of hepatocyte growth factor (HGF) was examined in rats. Considering the elimination mechanism for HGF, two approaches were used to construct a drug delivery system (DDS) for HGF to increase its plasma residence time while maintaining its biological activity. From the results of the present studies, I would like to propose the following conclusions: (i) Liver is the major clearance organ for HGF over a wide dose range, including tracer and pharmacological doses. (ii) Elimination of HGF from the circulation exhibits biphasic saturation: Both receptor-mediated endocytosis and another uptake mechanism, probably mediated by heparan-sulfate proteoglycan, in the liver are the high-affinity and low-affinity clearance sites, respectively. (iii) Both the formation of a heparin-HGF complex and protamine-pretreatment are promising approaches to developing a DDS for HGF. Heparin-HGF complex exhibits a much lower total body clearance and higher plasma residence time compared with HGF alone, while the complex has a lower affinity for the HGF receptor. Protamine pretreatment increases both the plasma residence of HGF and its biological activity, resulting in an increase in its pharmacological effect *in vivo*. Thus, the latter approach seems to be appropriate for the development of DDS for HGF.

Generally, the highly efficient elimination from the body might be one of the stumbling blocks to the development of cytokines as pharmaceutical agents. To overcome this problem, construction of a DDS using heparin and protamine as described above should be a

promising approach. It should also be noted that many other approaches may also be possible. For example, controlling the intracellular trafficking of the ligand and/or receptor may be one alternative. Receptor-mediated endocytosis and the subsequent lysosomal degradation is the predominant clearance mechanism for many types of cytokines. Therefore, one possible approach is to prevent such intracellular degradation.

Another stumbling block to the development of cytokines as therapeutic options may be their wide range of biological effects. For example, HGF has now been shown to be a mitogen, motogen, morphogen, and a tumor suppressor, for many types of endothelial, epithelial, and tumor cells. This implies that there are many possible pharmacological targets for HGF if it is used as a treatment. However, such a variety of biological effects may actually induce side-effects in clinical use. To overcome this problem, it might be possible to construct DDS which allow only one or two pharmacological effects to be exhibited. In addition, it is also important to know how such multi-functional cytokines act as specific factors to repair injured organs *in vivo* under pathological conditions. Possibly, the concentration of such multi-functional cytokines around the target cells is highly regulated by several unknown mechanisms *in vivo*. It may be important to develop of treatment philosophy for the use of cytokines in the treatment of pathphysiological conditions in patients.

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 high (Mw 18-23 kD, 185.5 units/mg) or low (Mw 4-6 kD) molecular weight was purchased from Sigma (St. Louis, MO). Protamine sulfate was obtained from Salmon roe from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Human recombinant HGF purified from a culture medium of C-127 cells transfected with plasmid containing human HGFcDNA (48,49) was radiolabeled with ^{125}I-Na by the chloramine-T method as described previously (30). The specific activity of ^{125}I-HGF thus prepared was 70-160 Ci/g. Epidermal growth factor (EGF) was supplied by Wakunaga Pharmaceutical Co., Ltd. (Hiroshima, Japan). ^{125}I-deoxyuridine was from New England Nuclear (Boston, MA).

Analysis of the time profiles of plasma disappearance of ^{125}I-HGF or heparin-^{125}I-HGF complex

Heparin that was dissolved in saline was incubated with ^{125}I-HGF (5 μCi, 0.6 pmol/kg body wt) for 50 min at 25°C. Heparin-^{125}I-HGF

mixture thus obtained was administered through the femoral vein and, at specified times, blood samples were withdrawn through the femoral artery as described previously (30). The trichloroacetic acid (TCA)-precipitable radioactivities in each sample were determined (30). The plasma concentration time profiles of the TCA-precipitable ^{125}I-HGF (C_p) were fitted to the following two-exponential equation by the use of a nonlinear iterative least squares method (30):

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

where Cp is the plasma concentration of TCA-precipitable radioactivity, and α and β are the apparent rate constants. The area under the plasma concentration time curve from time 0 to t ($AUC_{(0-t)}$) was calculated as:

$$AUC_{(0-t)} = \int_0^t Cp\, dt = A/(1-\exp(-\alpha t)) + B/(1-\exp(-\beta t)) \quad (2)$$

To check the effect of heparin on the recovery of ^{125}I-HGF by the TCA-precipitation technique, we did the following experiment: At 5 min after the intravenous administration of ^{125}I-HGF, plasma was collected, and heparin was added to the plasma sample to give a final heparin concentration of 0 - 1 mg/ml, followed by the determination of TCA-precipitable radioactivity. As a result, no difference was observed at any concentration of heparin, suggesting that heparin has no effect on the recovery by the TCA-precipitation technique.

Pharmacokinetic analysis of HGF or heparin-HGF complex

Heparin dissolved in saline was incubated with HGF for 50 min at 25°C. Under light ether anesthesia, HGF alone or the heparin-HGF mixture was administered through the penis vein and, at specified times, blood samples were withdrawn through the jugular vein without any

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 Eq. (1) using a nonlinear iterative least squares method (30). The area under the plasma concentration-time curve from time 0 to t ($AUC_{(0-t)}$) was calculated by Eq. (2). The area under the plasma concentration-time curve from time 0 to infinity (AUC), area under the moment curve (AUMC), the plasma clearance (CL_{plasma}), distribution volume of the central compartment (V_1), and steady-state distribution volume (V_{dss}) were calculated as:

$$AUC = A/\alpha + B/\beta \quad (3)$$

$$AUMC = A/\alpha^2 + B/\beta^2 \quad (4)$$

$$CL_{plasma} = Dose / AUC \quad (5)$$

$$V_1 = Dose / (A + B) \quad (6)$$

$$V_{dss} = Dose\ AUMC / (AUC)^2 \quad (7)$$

Effect of protamine injection on the plasma elimination of HGF

Under light ether anesthesia 1 μg/kg HGF was injected through the penis vein. At indicated times, blood was withdrawn through the left external jugular vein. At 3.5 min, 250 μl saline containing protamine (0-20 mg/kg) was also injected through the penis vein and blood samples were collected. The plasma concentration of HGF was determined by EIA.

Ligation of portal vein and hepatic artery

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 only laparatomies were performed without any ligation.

Measurements of the distribution of ^{125}I-HGF or heparin-^{125}I-HGF complex by several tissues

At 10 min after the intravenous administration of ^{125}I-HGF (5 μCi, 0.6 pmol/kg body wt) or heparin-^{125}I-HGF complex prepared as described above, the rats were sacrificed and the liver, adrenal, spleen, kidney, lung, and duodenum were excised. An aliquot of each tissue was weighed and counted (30). The tissue-to-plasma concentration ratio (Kp) per g tissue is obtained by:

$$Kp = X_T / Cp \quad (8)$$

where X_T is the amount of ^{125}I-HGF in the tissue at time t. The tissue distribution volume ($V_{d,tissue}$) per kg body wt can be obtained by multiplying Eq. (8) by tissue weight (Vi) with a dimension of g tissue/kg body wt:

$$V_{d,tissue} = Kp\ Vi \quad (9)$$

Liver perfusion method

The method reported previously (30) was used. Heparin dissolved in the perfusion 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 monohydrate (MES) at pH of 7.4 was incubated with ^{125}I-HGF (6 nCi/ml, 0.8 pM) for 50 min at 25°C and heparin-^{125}I-HGF complex was obtained. Sequential single-pass

perfusions of a perfusate containing ^{125}I-HGF (6 nCi/ml, 0.8 pM) alone, mixtures of heparin (0.1 mg/ml)-^{125}I-HGF, heparin (0.3 mg/ml)-^{125}I-HGF, heparin(1 mg/ml)-^{125}I-HGF, heparin(3 mg/ml)-^{125}I-HGF were done; and the TCA-precipitable radioactivities in the perfusate samples drawn from the inlet and outlet of the liver were determined (30). The steady-state hepatic extraction ratio (E_h) of ^{125}I-HGF in each perfusion was calculated based on the following equation after the concentration of TCA-precipitable radioactivity in the outflow (C_{out}) reached a plateau (> 5 min):

$$E_h = 1 - C_{out} / C_{in} \qquad (10)$$

where C_{in} is the concentration of TCA-precipitable radioactivity in the inflow.

Assay for DNA synthesis in primary cultured rat hepatocytes

Parenchymal hepatocytes were plated at a density of 1.25 x 10^5 cells/1.88 cm^2 and cultured for 3 hour for HGF or 24 hour for HGF and EGF as described previously (23). The non-attached cells were removed by washing and the culture medium containing protamine was applied to the monolayer. HGF or EGF was added 10 min after the addition of protamine. Then, 22 hour after HGF addition, ^{125}I-deoxyuridine was added and its incorporation for 6 hour was assayed as described previously (23). Cellular protein was determined by the method described by Bradford, using the Bio-Rad protein assay kit with BSA as a standard.

Assay for DNA synthesis in primary cultured rat hepatocytes with different exposure time to HGF

HGF was added to rat hepatocytes which had been cultured for 24 h. After the indicated times the monolayer was washed and further incubated in HGF-free medium. Twenty two hours after the HGF addition, ^{125}I-deoxyuridine was added and its incorporation for 6 hour was assayed.

Measurement of biological activity of heparin-HGF complex

Heparin-HGF complex was prepared by incubation as described above and then added to 24 h-cultured rat hepatocytes. Twenty-two hours later, ^{125}I-deoxyuridine incorporation for 6 hour was assayed.

In vitro binding study of crude ^{35}S-heparin to HGF

^{35}S-heparin (15-25 mCi/g, Amersham, Arlington Heights, IL) dissolved in the perfusion buffer was incubated with HGF (25 or 50 nM) for 50 min at 25°C. Total and unbound concentration of radioactivity was determined by the ultrafiltration with Mw limitation of 30 kD. The binding parameters were obtained by fitting all the data to the following equation using an iterative non-linear least-squares methods:

$$Cb = n_1 Pt\ Cf / (Kd_1 + Cf) + n_2 Pt\ Cf / (Kd_2 + Cf) + \alpha\ Pt\ Cf \qquad (11)$$

where Cb, Cf, n, Pt, Kd, and α were the concentration of heparin bound to HGF, unbound heparin concentration, the specific binding capacity, HGF concentration, dissociation constant, and proportional constant for a nonspecific binding, respectively. The subscriptions of 1 and 2 represent high and low affinity binding site, respectively. Mw of ^{35}S-heparin is assumed to be 10 kD.

In vitro binding of purified ^{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 x 1.5 cm I. D.) at a flow rate of 0.18 ml/min using phosphate-buffered saline as elution buffer (73). Blue dextran (Pharmacia, Uppsala, Sweden), fluorescein dextran with a molecular weight of 40 and 10 kDa (Cosmo-Bio, Tokyo, Japan), ^{14}C-inulin (New England Nuclear, Boston, MA), and ^3H_2O (New England Nuclear, Boston, MA) were also separately eluted as molecular weight markers. The volume of each fraction was 2.0 ml. Fractions 44 to 46 and 56 to 58 were collected as ^{35}S-heparin with a molecular weight of 21-23 kDa and 12-13 kDa, respectively. The molecular weight of the ^{35}S-heparin was determined from a plot of k_{av} versus the logarithm of the molecular weight of each marker where:

$$k_{av} = (V_e - V_o) / (V_t - V_o) \qquad (12)$$

The V_e, V_o, and V_t were peak fraction numbers for each molecular weight marker, blue dextran, and ^3H_2O, respectively. ^{35}S-heparin dissolved in the perfusion buffer was incubated with HGF (50 nM) for 50 min at 25°C. Total and unbound radioactivity was determined by ultrafiltration with an Mw limitation of 30 kDa. The binding parameters were obtained by fitting the data to the following equation using an iterative non-linear least-squares method:

$$C_b = n\ Pt\ C_f / (K_d + C_f) + \alpha\ Pt\ C_f \qquad (13)$$

where C_b, C_f, n, Pt, 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.

Simulation of the occupation of heparin binding sites on HGF

The binding parameters obtained in the present study were used in the simulation study. The total heparin concentration (Ct) was fixed to 1 mg/ml. For the varied HGF concentration (Pt), Cb and Cf were obtained using Eq. (11) and the following equation:

$$Ct = Cb + Cf \quad (14)$$

The occupancies (%) of high and low affinity heparin binding sites on HGF molecule were calculated as:

$$\%OCCUPANCY = Cf / (Kd_1 + Cf) \times 100 \text{ (high affinity site)}$$

$$\%OCCUPANCY = Cf / (Kd_2 + Cf) \times 100 \text{ (low affinity site)} \quad (15)$$

HGF and protamine injection to the liver-injured rats

α-naphthylisothiocyanate (ANIT) dissolved in olive oil was injected intraperitoneally at a dose of 50 mg/kg body wt. While rats were under ether anesthesia, partial (30%) hepatectomy was performed by removing the left lateral lobe of the liver through a subxyphoid incision. HGF dissolved in saline was administered through the penis vein 30 min before and 8, 22, 32, 46, 56, 70, 80, 94, 104, and 118 hour after ANIT treatment or 8, 22, 32, 46 hour after partial hepatectomy. Rats were sacrificed 12, 24, 48, 72, 96, and 120 hour after ANIT treatment or 48 hour after 30 % partial hepatectomy. Protamine dissolved in saline was administered through the penis vein 10 min before HGF or EGF injection.

Measurement of labeling index

One hour before sacrificing the rats, 5-bromo-2'-deoxyuridine dissolved in normal saline was injected intraperitoneally, 100 mg/kg body

wt. One hour after injection, the rats were exsanguinated via the abdominal artery under light ether anethesia. The liver was then removed and fixed in 10% buffered formalin for 24 hour. The fixed samples were embedded in paraffin and the paraffin sections (4 μm) mounted on a glass slide. After deparaffinization of the liver sections, endogenous peroxidase was inactivated in 0.3 % hydrogen peroxide in absolute methanol and nuclei incorporating 5-bromo-2'-deoxyuridine were stained using a Cell Proliferation Kit from Amersham (Arlington Heights, IL). The labeling index of hepatocytes was determined by counting more than 500 nuclei in photographs of three randomly selected fields under light microscopy.

Determination of bilirubin concentration and activity of liver cytosolic enzymes in serum

The total bilirubin concentration (BIL) and the activity of liver-specific cytosolic enzymes such as glutamic-pyruvic transaminase (GPT), lactate dehydrogenase (LAP), alkaline phosphatase (ALP) and γ-glutamyltransferase (γ -GTP) in rat serum obtained 48 hour after ANIT treatment were determined using the appropriate assay kits (Wako Pure Chemical Industries, Osaka, Japan).

Pharmacokinetic analysis of HGF in ANIT-treated rats

Under light ether anesthesia, protamine (0 or 1.6 mg/kg) was administered through the penis vein to rats 24 hour after ANIT treatment. Ten minutes after the protamine injection, HGF (300 μg/kg body wt) dissolved in saline was also given through the penis vein.

Plasma was collected from the external jugular vein and the HGF concentration was determined using an EIA kit.

Elution of HGF by protamine using heparin-sepharose column chromatography

1.0 ml HGF (250 ng/ml) dissolved in PBS was added to a heparin column (1 ml bed volume, heparin-sepharose CL 6B, Pharmacia) at the rate of 0.3 ml/min and incubated for 30 min on ice. Then, 1.0 ml PBS or 1.0 ml protamine (20 mg/ml) was applied to the column 13 times at the same rate. Finally, 1.0 ml PBS containing 2 M sodium chloride was added to the column 6 times. Each eluted solution was collected to determine HGF by EIA. The ratio of the amount of HGF eluted in each sample to that added to the column was calculated as the recovery of HGF.

Statistical analysis

Statistical analysis was performed by Student's t-test to identify significant differences between various treatment groups.

REFERENCES

1. Ando, T., M. Yamasaki, and K. Suzuki. Protamines. In: *Molecular Biology Biochemistry and Biophysics*. A. Kleinzeller, G. F. Springer, and H. G. Wittmann (Eds.), Springer-Verlag, Berlin, Vol. 12: p. 1-30, 1973.
2. Asami, O., I. Ihara, N. Shimidzu, S. Shimizu, Y. Tomita, A. Ichihara, amd T. Nakamura. Purification and characterization of hepatocyte growth factor from injured liver of carbon tetrachloride-treated rats. *J. Biochem.* 109: 8-13, 1991.
3. Bardelli, A., C. Ponzetto, and P. M. Comoglio. Identification of functional domains in the hepatocyte growth factor and its receptor by molecular engineering. *J. Biotechnol.* 37: 109-122, 1994.
4. Bottaro, D. P., J. S. Rubin, D. L. Faletto, A. Chan, T. E. Kmiecik, G. F. Vande Woude, and S. A. Aaronson. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science* 251: 802-804, 1991.
5. Burwen, S. J., M. E. Barker, I. S. Goldman, G. T. Hradek, S. E. Raper, and A. L. Jones. Transport of epidermal growth factor by rat liver: evidence for a nonlysosomal pathway. *J. Cell. Biol.* 99: 1259-1265, 1984.
6. Bussolino, F., M. F. Di Renzo, M. Ziche, E. Bocchietto, M. Olivero, L. Naldini, G. Gaudino, L. Tamagnone, A. Coffer, and P. M. Comoglio. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J. Cell Biol.* 119: 629-641, 1992.

7. Dunn, W. A. and A. L. Hubbard. Receptor-mediated endocytosis of epidermal growth factor by hepatocytes in the perfused rat liver: ligand and receptor dynamics. *J. Cell Biol.* 98: 2148-2159, 1984.

8. Francavilla, A., A. Azzarone, G. Carrieri, C. S. Foglieni, Q. H. Zeng, U. Cillo, K. Porter, and T. E. Starzl. Effect of the canine Eck fistula liver of intraportal TGF-β alone or with hepatic growth factors. *Hepatology* 16: 1267-1270, 1992.

9. Francavilla, A., T. E. Starzl, K. Porter, C. S. Foglieni, G. K. Michalopoulos, G. Carrieri, J. Trejo, A. Azzarone, M. Barone, and Q. H. Zeng. Screening for candidate hepatic growth factor by selective portal infusion after canine Eck's fistula. *Hepatology* 14: 665-670, 1991.

10. Gohda, E., H. Tsubouchi, H. Nakayama, S. Hirono, O. Sakiyama, K. Takahashi, H. Miyazaki, S. Hashimoto, and Y. Daikuhara. Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. *J. Clin. Invest.* 81: 414-419, 1988.

11. Gospodarowicz, D. and J. Cheng Heparin protects basic and acidic FGF from inactivation. *J. Cell Physiol.* 128: 475-484, 1986.

12. Green, D., J. Hirsh, J. Heit, M. Prins, B. Davidson, and A. W. Lensing. Low molecular weight heparin: a critical analysis of clinical trials. *Pharmacol. Rev.* 46: 89-109, 1994.

13. Heldin, C. H. Dimerization of cell surface receptors in signal transduction. *Cell* 80: 213-223, 1995.

14. Higuchi, O., K. Mizuno, G. F. Vande Woude, and T. Nakamura. Expression of c-met proto-oncogene in COS cells induces the signal

transducing high-affinity receptor for hepatocyte growth factor. *FEBS Lett.* 301: 282-286, 1992.

15. Higuchi, O. and T. Nakamura. Identification and change in the receptor for hepatocyte growth factor in rat liver after partial hepatectomy or induced hepatitis. *Biochem. Biophys. Res. Commun.* 176: 599-607, 1991.
16. Huang, J. S., J. Nishimura, S. S. Huang, and T. Deuel. Protamine inhibits platelet derived growth factor receptor activity but not epidermal growth factor activity. *J. Cell. Biochem.* 26: 205-220, 1984.
17. Igawa, T., K. Matsumoto, S. Kanda, Y. Saito, and T. Nakamura. growth factor may function as a renotropic factor for regeneration in rats with acute renal injury. *Am. J. Physiol.* 265: F61-F69, 1993.
18. Ishibashi, K., S. Sasaki, H. Sakamoto, Y. Nakamura, T. Hata, T. Nakamura, and F. Marumo. Hepatocyte growth factor is a paracrine factor for renal epithelial cells: stimulation of DNA synthesis and Na, K-ATPase activity, *Biochem. Biophys. Res. Commun.* 182: 960-965, 1992.
19. Ishiki, Y., H. Ohnishi, Y. Muto, K. Matsumoto, and T. Nakamura. Direct evidence that hepatocyte growth factor is a hepatotrophic factor for liver regeneration and for potent anti-hepatitis action *in vivo*. *Hepatology* 16: 1227-1235, 1992.
20. Jaques, L. B. Protamin-antagonist to heparin. *J. Can. Med. Assoc.* 108: 1291-1297, 1973.
21. Kato, Y., K. Liu, T. Nakamura, and Y. Sugiyama. Heparin-hepatocyte growth factor complex with low plasma clearance and retained hepatocyte proliferating activity. *Hepatology* 20: 417-424, 1994.

22. Kato, Y., H. Sato, M. Ichikawa, H. Suzuki, Y. Sawada, M. Hanano, T. Fuwa, and Y. Sugiyama. Existence of phenylarsine oxide sensitive and -insensitive pathways for the receptor-mediated endocytosis of epidermal growth factor by rat liver. *Proc. Natl. Acad. Sci. USA* 89: 8507-8511, 1992.

23. Kato, Y. and Y. Sugiyama Y. Binding, internalization, degradation, and mitogenic effect of epidermal growth factor in cultured rat hepatocytes. *STP Pharm. Sci.* 3: 75-82, 1993.

24. Sugiyama, Y. and Y. Kato. *In vitro* models of hepatic uptake: Methods to determine kinetic parameters for receptor-mediated hepatic uptake. In: *Peptide-Based Drug Design: Controlling Transport and Metabolism*. M. D. Taylor and G. L. Amidon (Eds.), American Chemical Society Books Department, Washington, DC, USA, p. 525-551, 1995.

25. Kawaida, K., K. Matsumoto, H. Shimazu, and T. Nakamura. Hepatocyte growth factor prevents acute renal failure and accelerates renal regeneration in mice. *Proc. Natl. Acad. Sci.* USA 91: 4357-4361, 1994.

26. Kinoshita, T., K. Tashiro, and T. Nakamura. Marked increase of HGF mRNA in non-parenchymal liver cells of rats treated with hepatotoxins. *Biochem. Biophys. Res. Commun.* 165: 1229-1234, 1989.

27. Kobayashi, Y., M. Hamanoue, S. Ueno, T. Aikou, G. Tanabe, S. Mitsue, K. Matsumoto, and T. Nakamura. Induction of hepatocyte growth by intraportal infusion of HGF into beagle dogs. *Biochem. Biophys. Res. Commun.* 220: 7-12, 1996.

28. Lee, C. C. and K. M. Yamada. Alternatively spliced juxtamembrane domain of a tyrosine kinase receptor is a multifunctional regulatory

site. Deletion alters cellular tyrosine phosphorylation pattern and facilitates binding of phosphatidylinositol-3-OH kinase to the hepatocyte growth factor receptor. *J. Biol. Chem.* 270: 507-510, 1995.

29. Lindroos, P. M., R. Zarnegar, and G. K. Michalopoulos. Hepatocyte growth factor(Hepatopoietin A) rapidly increase in plasma before DNA synthesis and liver regeneration stimulated by partial hepatectomy and carbon tetrachloride administration. *Hepatology* 13: 743-749, 1991.
30. Liu, K., Y. Kato, M. Narukawa, D. C. Kim, M. Hanano, O. Higuchi, T. Nakamura, and Y. Sugiyama. The importance of the liver in the plasma clearance of hepatocyte growth factor in rats. *Am. J. Physiol.* 263: G642-G649, 1992.
31. Liu, K., Y. Kato, M. Yamazaki, O. Higuchi, T. Nakamura, and Y. Sugiyama. Decrease in the hepatic uptake clearance of hepatocyte growth factor in CCl_4-intoxicated rats. *Hepatology* 17: 651-660, 1993.
32. Liu, K. X., Y. Kato, T. Terasaki, S. Aoki, K. Okumura, T. Nakamura, and Y. Sugiyama. Contribution of parenchymal and non-parencymal liver cell to the clearance of hepatocyte growth factor from the circulation in rats. *Pharm. Res.* 12: 1737-1740, 1995.
33. Liu, K., Y. Kato, T. Terasaki, T. Nakamura, and Y. Sugiyama. Change in hepatic handling of hepatocyte growth factor during liver regeneration in rats. *Am. J. Physiol.* 269: G745-G753, 1995.
34. Lokeshwar,V. B., S. S. Huang, and J. S. Huang. Protamine enhances epidermal growth factor (EGF)-stimulated mitogenesis by increasing cell surface EGF receptor number. *J. Biol. Chem.* 264: 19318-19326, 1989.

35. Lyon, M., J. A. Deakin, and J. T. Gallagher. Liver heparan sulfate structure. *J. Biol. Chem.* 269: 11208-11215, 1994.

36. Lyon, M., J. A. Deakin, K. Mizuno, T. Nakamura, and J. T. Gallagher. Interaction of hepatocyte growth factor with heparan sulfate. *J. Biol. Chem.* 269: 11216-11223, 1994.

37. Maack, T., M. Suzuki, F. A. Almeida, D. Nussenzveig, R. M. Scarborough, G. A. McEnroe, and J. A. Lewicki. Physiological role of silent receptors of atrial natriuretic factor. *Science* 238: 675-678, 1987.

38. Masumoto, A. and N. Yamamoto. Sequestration of a hepatocyte growth factor in extracellular matrix in normal adult rat liver. *Biochem. Biophys. Res. Commun.* 174: 90-95, 1991.

39. Matsumoto, K., H. Tajima, M. Hamanoue, S. Kohno, T. Kinoshita, and T. Nakamura. Identification and characterization of "injurin," an inducer of expression of the gene for hepatocyte growth factor. *Proc. Natl. Acad. Sci. USA* 89: 3800-3804, 1992.

40. Matsumoto, K., K. Hashimoto, K. Yoshikawa, and T. Nakamura. Marked stimulation of growth and motility of human keratinocytes by hepatocyte growth factor. *Exp. Cell Res.* 196: 114-120, 1991.

41. Matsumoto, K. and T. Nakamura. Emerging multipotent aspects of hepatocyte growth factor. *J. Biochem.* 119: 591-600, 1996.

42. Matsumoto, K. and T. Nakamura. Hepatocyte growth factor: Molecular structure, roles in liver regeneration, and other biological functions. *Critical Rev. in Oncogenesis.* 3: 27-54, 1992.

43. Mizuno, K., H. Inoue, M. Hagiya, S. Shimizu, T. Nose, Y. Shimohigashi, and T. Nakamura. Hairpin loop and second kringle

domain are essential sites for heparin binding and biological activity of hepatocyte growth factor. *J. Biol. Chem.* 269: 1131-1136, 1994.

44. Montesano, R., K. Matsumoto, T. Nakamura, and L. Orci. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* 67: 901-908, 1991.
45. Naka, D., T. Ishii, T. Shimomura, T. Hishida, and H. Hara. Heparin modulates the receptor-binding and mitogenic activity of hepatocyte growth factor on hepatocytes. *Exp. Cell Res.* 209: 317-324, 1993.
46. Nakamura, T., H. Teramoto, and A. Ichihara. Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary cultures. *Proc. Natl. Acad. Sci. USA* 83: 6489-6493, 1986.
47. Nakamura, T., K. Nawa, and A. Ichihara. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem. Biophys. Res. Commun.* 122: 1450-1459, 1984.
48. Nakamura, T., K. Nawa, A. Ichihara, A. Kaise, and T. Nishino. Purification and subunit structure of hepatocyte growth factor from rat platelets. *FEBS Lett.* 224: 311-316, 1987.
49. Nakamura, T., T. Nishizawa, M. Hagiya, T. Seki, M. Shimonishi, A. Sugimura, K. Tashiro, and S. Shimizu. Molecular cloning and expression of human hepatocyte growth factor. *Nature* 342: 440-443, 1989.
50. Noji, S., K. Tashiro, E. Koyama, T. Nohno, K. Ohyama, S. Taniguchi, and T. Nakamura. Expression of hepatocyte growth factor gene in endothelial and kupffer cells of damaged rat livers, as

revealed by in situ hybridization. *Biochem. Biophys. Res. Commun.* 173: 42-47, 1990

51. Ohmichi, H., K. Matsumoto, and T. Nakamura. *In vivo* mitogenic action of HGF on lung epithelial cells: pulmotrophic role in lung regeneration. *Am. J. Physiol.* 270: L1031-L1039, 1996.
52. O'Reilly, R. A. Anticoagulant, antithrombotic, and thrombolytic drugs. In: *The pharmalogical Basis of Therapeutics.* A.G. Goodman, L. S. Gilman, and A. Gilman (Eds.), Macmillan, New York, p. 1347-1366, 1980.
53. Prat, M., R. P. Narsimhan, T. Crepaldi, M. R. Nicotra, P. G. Natali, and P. M. Comoglio. The receptor encoded by the human c-met oncogene is expressed in hepatocytes, epithelial cells and solid tumors. *Int. J. Cancer* 49: 323-328, 1991.
54. Ramadori, G., K. Neubauer, M. Odenthal, T. Nakamura, T. Knittel, S. Schwogler, and K. H. M. Buschenfelde. The gene of hepatocyte growth factor is expressed in fat-storing cells of rat liver and is downregulated during cell growth and by transforming growth factor-β. *Biochem. Biophys. Res. Commun.* 183: 739-742, 1992.
55. Rapraeger, A. C., A. Krufka, and B. B. Olwin. Requirment of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* 252: 1705-1708, 1991.
56. Roos, F., A. M. Ryan, S. M. Chamow, G. L. Bennett, and R. H. Schwall. Induction of liver growth in normal mice by infusion of hepatocyte growth factor/scatter factor. *Am. J. Physiol.* 268: G380-G386, 1995.
57. Rosengart, T. K., W. V. Johnson, R. Friesel, R. Clark, and T. Maciag. Heparin protects heparin-binding growth factor-I from

proteolytic inactivation *in vitro*. *Biochem. Biophys. Res. Commun.* 152: 432-440, 1988.

58. Rubin, J. S., D. P. Bottaro, and S. A. Aaronson. Hepatocyte growth factor/scatter factor and its receptor, the c-met proto-oncogene product. *Biochem. Biophys. Acta.* 1155: 357-371, 1993.
59. Ruoslahti, E. and Y. Yamaguchi. Proteoglycans as modulators of growth factor activities. *Cell* 64: 867-869, 1991.
60. Sacks, D. B., and J. M. McDonald. Insulin-stimulated phosphorylation of calmodulin by rat liver insulin receptor preparations. *J. Biol. Chem.* 263: 2377-2383, 1988.
61. Sato, H., Y. Sugiyama, Y. Sawada, T. Iga, S. Sakamoto, T. Fuwa, and M. Hanano. Dynamic determination of kinetic parameters for the interaction between polypeptide hormones and cell-surface receptors in the perfused rat liver by the multiple-indicator dilution method. *Proc. Natl. Acad. Sci. USA* 85: 8355-8359, 1988.
62. Sato, H., Y. Sugiyama, Y. Sawada, T. Iga, T. Fuwa, and M. Hanano. Internalization of EGF in perfused rat liver is independent of the degree of receptor occupancy. *Am. J. Physiol.* 258: G682-G689, 1990.
63. Schwall, R. H., L. Y. Chang, P. J. Godowski, D.W. Kahn, K. J. Hillan, K. D. Bauer, and T. F. Zioncheck. Heparin induces dimerization and confers proliferative activity onto the hepatocyte growth factor antagonists NK1 and NK2. *J. Cell Biol.* 133: 709-718, 1996.
64. Shiota, G., T. C. Wang, T. Nakamura, and E. V. Schmidt. Hepatocyte growth factor in transgenic mice: effect on hepatocyte growth, liver regeneration and gene expression. *Hepatology* 19: 962-972. 1994.

65. Sugiyama, Y., D. C. Kim, H. Sato, S. Yanai, H. Satoh, T. Iga, and M. Hanano. Receptor-mediated disposition of polypeptides: Kinetic analysis of the transport of epidermal growth factor using *in vitro*, isolated perfused organs, and *in vivo* system. *J. Control Rel.* 1990; 13: 157-174.

66. Sugiyama, Y, and M. Hanano. Receptor-mediated transport of peptide hormones and its importance in the overall hormone disposition in the body. *Pharm. Res.* 6: 194-204, 1989.

67. Tajima, H., O. Higuchi, K. Mizuno, and T. Nakamura. Tissue distribution of hepatocyte growth factor receptor and its exclusive down-regulation in a regenerating organ after injury. *J. Biochem.* 111: 401-406, 1992.

68. Takehara, T., K. Matsumoto, and T. Nakamura. Cell density-dependent regulation of albumin synthesis and DNA synthesis in rat hepatocytes by hepatocyte growth factor. *J. Biochem.* 112: 330-334, 1992.

69. Tashiro, K., M. Hagiya, T. Nishizawa, T. Seki, M. Shimonishi, S. Shimizu, and T. Nakamura. Deduced primary structure of rat hepatocyte growth factor and expression of the mRNA in rat tissues. *Proc. Natl. Acad. Sci. USA* 87: 3200-3204, 1990.

70. Turnbull, J. E., D. G. Fering, Y. Ke, M. C. Wilkinson, and J. T. Gallagher. Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate. *J. Biol. Chem.* 267: 10287-10293, 1992.

71. Vigna, E., L. Naldini, L. Tamagnone, P. Longati, A. Bardelli, F. Maina, C. Ponzetto, and P. M. Comoglio. Hepatocyte growth factor

and its receptor, the tyrosine kinase encoded by the c-MET proto-oncogene. *Cell Mol. Biol.* 40: 597-604, 1994.

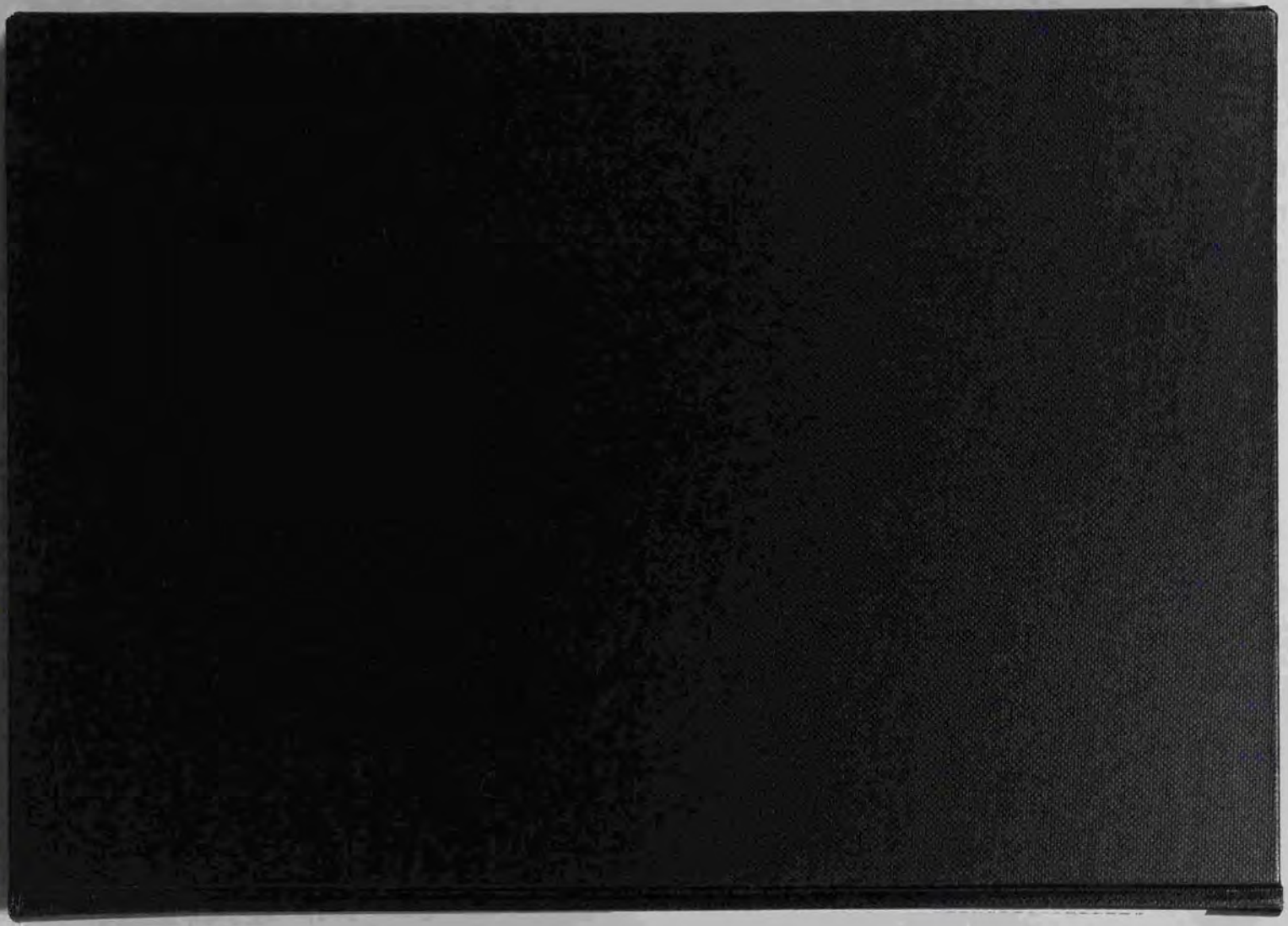
72. Wakefield, T. W., C. B. Hantler, S. K. Wrobleski, B. A. Crider, and J. C. Stanley. Effect of differing rats of protamine reversal of heparin anticoagulation. *Surgery* 119: 123-128, 1996.
73. Watanabe, J., K. Hori, K. Iwamoto, and S. Ozeki. Disposition of tritium-labelled heparin in rats. *J. Pharm. Dyn.* 5: 627-637, 1982.
74. Weidner, K. M., M. Sachs, and W. Birchmeier. The Met receptor tyrosine kinase transducers motility, proliferation, and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells. *J. Cell Biol.* 121: 145-154, 1993.
75. Weidner, K. M., N. Arakaki, J. Vandekerchove, S. Weingart, G. Hartmann, H. Rieder, C. Fonatsch, H. Tsubouchi, T. Hishida, Y. Daikuhara, and W. Birchmeier. Evidence for the identity of human scatter factor and hepatocyte growth factor. *Proc. Natl. Acad. Sci. USA* 88: 7001-7005, 1991.
76. Yanagita, K., M. Nagaike, H. Ishibashi, Y. Niho, K. Matsumoto, and T. Nakamura. Lung may have an endocrine function producing hepatocyte growth factor in response to injury of distal organs. *Biochem. Biophys. Res. Commun.* 182: 802-809, 1992.
77. Yanai, S., Y. Sugiyama, T. Iga, T. Fuwa, and M. Hanano. Kinetic analysis of receptor-mediated endocytosis of epidermal growth factor by isolated rat hepatocytes. *Am. J. Physiol.* 260: C457-C467, 1991.
78. Yanai, S., Y. Sugiyama, T. Iga, T. Fuwa, and M. Hanano. Kinetic analysis of the down-regulation of epidermal growth factor receptors in rats *in vivo*. *Am. J. Physiol.* 258: C593-C598, 1990.

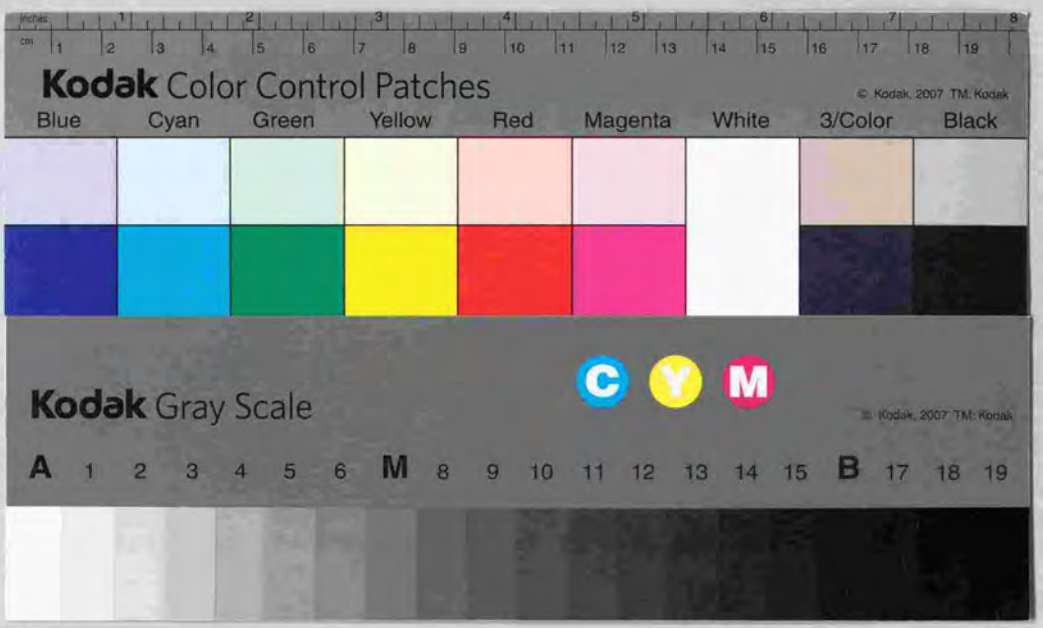
79. Zarnegar, R. and G. Michalopoulos. Purification and biological characterization of human hepatopoietin A, a polypeptide growth factor for hepatocytes. *Cancer Res.* 49: 3314-3320, 1989.

80. Zarnegar, R., M. C. DeFrances, D. P. Kost, P. M. Lindroos, and G. K. Michalopoulos. Expression of hepatocyte growth factor mRNA in regenerating rat liver after partial hepatectomy. *Biochem. Biophys. Res. Commun.* 177: 559-565, 1991.

81. Zarnegar, R., M. C. DeFrances, L. Oliver, and G. Michalopoulos. Identification and partial characterization of receptor binding sites for HGF on rat hepatocytes. *Biochem. Biophys. Res. Commun.* 173: 1179-1185, 1990.

82. Zioncheck, T. F., L. Richardson, J. Liu, L. Chang, K. L. King, G. L. Bennett, P. Fugedi, S. M. Chamow, R. H. Schwall, and R. J. Stack. Sulfated oligosaccharides promote hepatocyte growth factor association and govern its mitogenic activity. *J. Biol. Chem.* 270:16871-16878, 1995.

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