Protamine enhances the proliferative activity of hepatocyte growth factor in rats

Ke-Xin Liu[1)], Yukio Kato[1)], Tai-ichi Kaku[2)], Kunio Matsumoto[3)] , Toshikazu Nakamura[3)] ,and Yuichi Sugiyama[1)] *

[1)] Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, [2)] Japan Bioproducts Industry Co., Ltd., Tomigaya, Shibuya-ku, Tokyo 151, and [3)] Biomedical Research Center, Osaka University School of Medicine, Suita, Osaka 565, Japan

* To whom correspondence should be addressed.
Address all correspondence to:
Professor Yuichi Sugiyama, Ph. D.
Faculty of Pharmaceutical Sciences,
University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113 (Japan)
TEL: +81-3-5689-8094
FAX: +81-3-5800-6949

INDEX TERMS
Hepatocyte growth factor (HGF), Protamine, Liver regeneration

RUNNING TITLE

Protamine enhances liver regeneration induced by HGF

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, 1) lowering the overall elimination of HGF and 2) 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 (18). HGF stimulates proliferation of a variety types of epithelial cells including hepatocytes (6, 8, 16, 20). Its gene expression is increased not only when there is hepatic damage, such as partial hepatectomy (25, 31) and carbon tetrachloride poisoning (10), but also following renal (5) and pulmonary injury (20). In such cases HGF levels in circulating plasma are increased and, therefore, HGF is believed to be a hepatotrophic, renotrophic, and pulmotrophic factor (5, 20, 31).

HGF is a basic polypeptide and one of the heparin binding proteins (2, 19). HGF can bind to heparan sulfate expressed on the surface of ubiquitous cells and in the extracellular matrix (15, 32). 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 (17). 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 (26). 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 (24). This effect may result from stabilization

of the HGF dimer, which stimulates dimerization of the HGF receptor on the cell surface (24).

HGF markedly accelerates regeneration of damaged organs in experimental animals with hepatic and renal failure (6). However, a large dose (> 100 μg/kg) is usually required to exert such a pharmacological effect (4). 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 (11-14), 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 (11-14). When HGF is premixed with heparin and then given intravenously, its plasma clearance is reduced (8, 14). 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 (8, 11-14). However, high concentrations (>100 μg/ml) of heparin reduces the mitogenic activity of HGF (8). 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. 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.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250 g (Nisseizai, Tokyo, Japan) were used and treated humanely. The studies reported in this manuscript have been carried out in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. 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.

Materials

Protamine sulfate was obtained from Salmon roe from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); ANIT and 3,3'-diaminobenzidine were from Sigma (St. Louis, MO) and ^{125}I-deoxyuridine was from New England Nuclear (Boston, MA). Human recombinant HGF was purified from a culture medium of C-127 cells transfected with plasmid containing human HGFcDNA (18). Epidermal growth factor (EGF) was supplied by Wakunaga Pharmaceutical Co., Ltd. (Hiroshima, Japan).

HGF injection

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 h after ANIT treatment or 8, 22, 32, 46 h after partial hepatectomy. Rats were sacrificed 12, 24, 48, 72, 96, and 120 h after ANIT treatment or 48 h after 30 % partial hepatectomy.

Protamine injection

Under light ether anesthesia, 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 h. 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 h 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 h 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 enzyme-immuno assay (EIA) kit (Institute of Immunology, Tochigi, Japan).

The plasma concentration (Cp) - time profiles of HGF after intravenous administration were fitted to the following two-exponential equation by a nonlinear iterative least squares method (11):

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

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

The area under the plasma concentration-time curve (AUC) and area under the moment curve (AUMC) were calculated as:

$$AUC = A/\alpha + B/\beta \quad (2)$$

$$AUMC = A/\alpha^2 + B/\beta^2 \quad (3)$$

The plasma clearance (CL_{plasma}), distribution volume of the central compartment (V_1), and steady-state distribution volume (V_{dss}) were calculated from Eqs. 4, 5, and 6, respectively:

$$CL_{plasma} = Dose / AUC \quad (4)$$

$$V_1 = Dose / (A + B) \quad (5)$$

$$V_{dss} = Dose\ AUMC / (AUC)^2 \quad (6)$$

Effect of protamine injection on the plasma elimination of HGF

Under light ether anesthesia 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 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.

Assay for DNA synthesis in primary cultured rat hepatocytes

Parenchymal hepatocytes were plated at a density of 1.25×10^5 cells/1.88 cm^2 and cultured for 3 h for HGF or 24 h for HGF and EGF as described previously (8). 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 h after HGF addition, ^{125}I-deoxyuridine was added and its incorporation for 6 h was assayed as described previously (8). Cellular protein was determined by the method described by Bradford, using the Bio-Rad protein assay kit with BSA as a standard (9).

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.

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. 1). 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 h after ANIT treatment, respectively (Fig. 1). 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. 1). 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. 1).

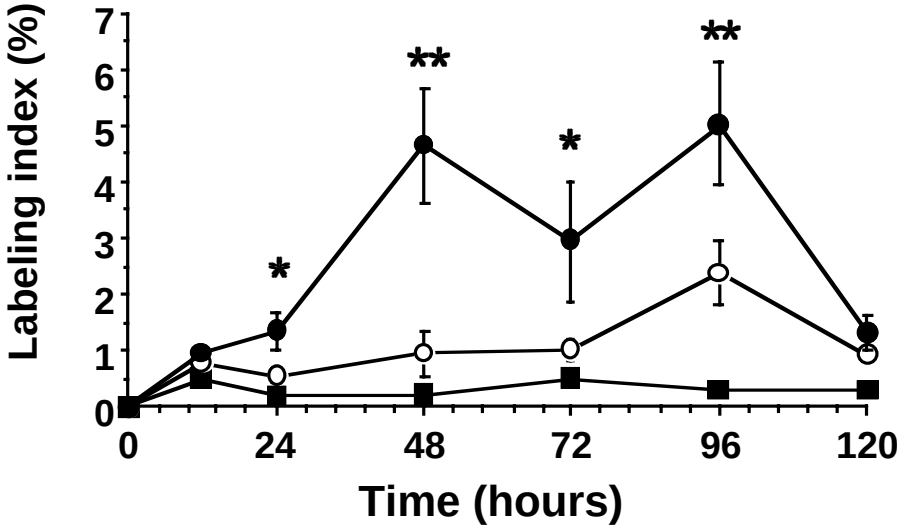

Fig. 1 Time-profiles of DNA synthesis in hepatocytes of ANIT-intoxicated rats treated with HGF alone or protamine prior to HGF injection

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 ± S.E. of 3-6 rats. *Significantly different from HGF alone ($p < 0.05$); ** ($p < 0.01$).

To examine the protamine dose-dependence, a similar experiment with various doses of protamine was performed and the labeling indices were determined 48 h after ANIT intoxication (Fig. 2A). Protamine alone could not stimulate liver regeneration in ANIT-intoxicated rats (Fig. 2A). 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. 2A). When the dose of protamine was further increased to over 1.6 mg/kg, a dose-dependent reduction in labeling index was observed (Fig. 2A). 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. 2A). An enhancing effect of protamine on liver regeneration induced by HGF was also found in partially (30%) hepatectomized rats (Fig. 2B). The protamine dose-dependence in the labeling indices was similar to that in ANIT-intoxicated rats (Fig. 2A, 2B). 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. 2B).

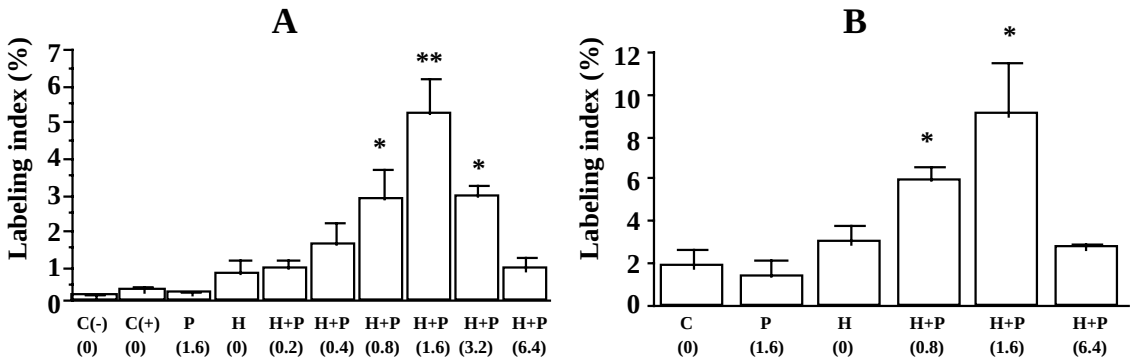

Fig. 2 Effect of protamine on the liver regeneration induced by HGF (A) in ANIT-intoxicated, as well as partially hepatectomized (B), rats.

ANIT-intoxicated rats were treated with HGF (300 μg/kg) alone (H), or various doses of protamine prior to the injection of HGF (H+P), EGF (E+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 ± S.E. of 3-5 rats. *Significantly different from HGF alone ($p < 0.05$); ** ($p < 0.01$).

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 function 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. 3). Protamine alone did not reduce the BIL or the activity of liver cytosolic enzymes in serum (Fig. 3). 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. 3). 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. 3). Protamine slightly enhanced the reduction produced by HGF in BIL, GPT, and LAP although this effect was not significant (Fig. 3).

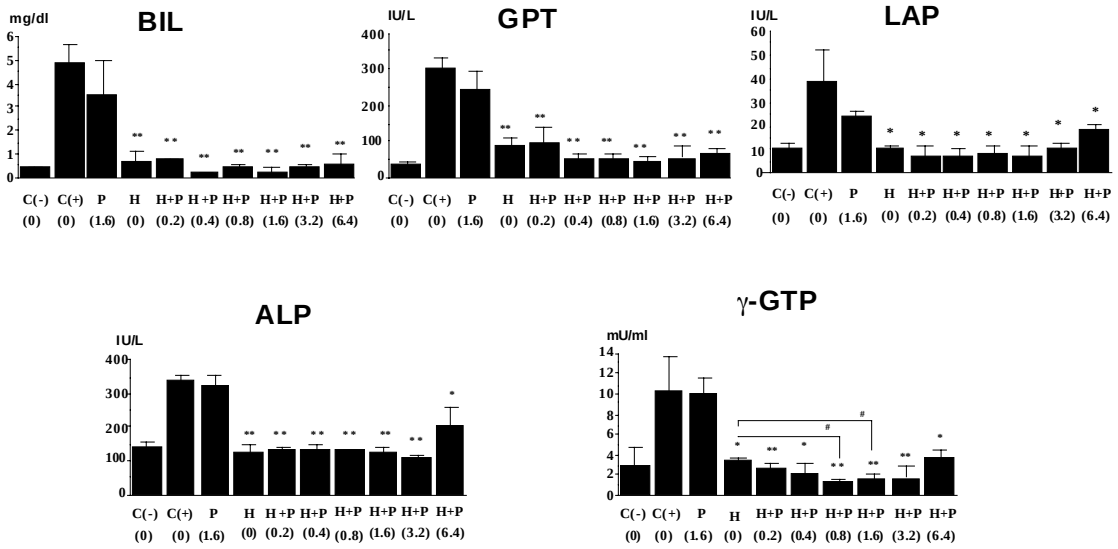

Fig. 3 Change in bilirubin concentration and activity of liver cytosolic enzymes in serum in ANIT-intoxicated rats treated with HGF alone or protamine prior to the injection of HGF

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 ± S.E. of 3 rats. *Significantly diff erent from C(+). ($p < 0.05$); ** ($p < 0.01$); #Significantly different from HGF alone ($p < 0.05$).

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. 4). The elimination of HGF from plasma after injection of HGF following protamine treatment was slower, compared with that after administration of HGF alone (Fig. 4). The AUC after administration of HGF following protamine injection was 2.48-fold that after HGF injected alone (Table 1). 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 1).

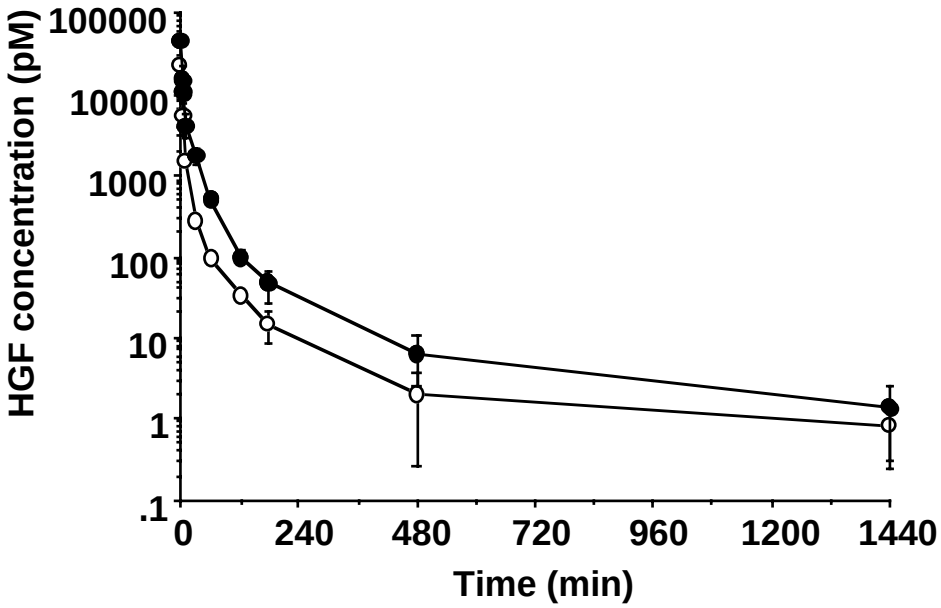

Fig. 4 Effect of protamine on the pharmacokinetics of HGF in ANIT-intoxicated rats

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 ± S.E. of 3 rats.

Table 1. Comparison of the pharmacokinetic parameters of HGF in ANIT-intoxicated ra 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 (300μg/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	$2.36 \times 10^3 \pm 1.49 \times 10^3$

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. 5). The labeling index at 300 μg/kg HGF

following 1.6 mg/kg protamine treatment was 4.63 ± 1.01 %, much higher than that after administration of HGF alone at dose of 500 μg/kg (0.530 ± 0.104 %) (Fig. 5) although in both cases the AUC had almost the same value (Fig. 5).

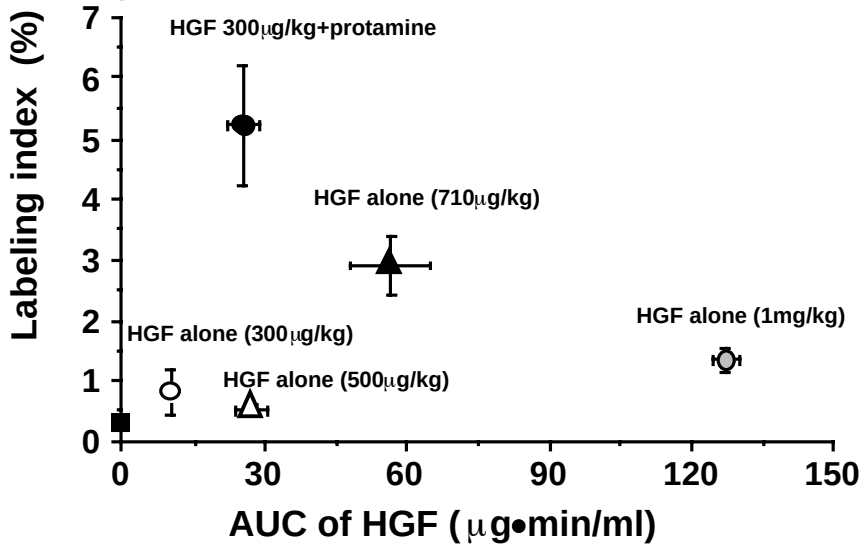

Fig. 5 Relationship between HGF AUC and liver regeneration in ANIT-intoxicated rats

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 ± S.E. of 3-5 rats.

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. 6A, 6B). 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. 6A, 6B). 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. 6B). 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. 6A, 6B). To examine whether the enhancing effect of protamine is specific to HGF, we performed the same experiment with EGF (Fig. 6C). 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. 6C). When the concentration of protamine was increased to 50 and 200 μg/ml, the DNA synthesis of hepatocytes was inhibited (Fig. 6C).

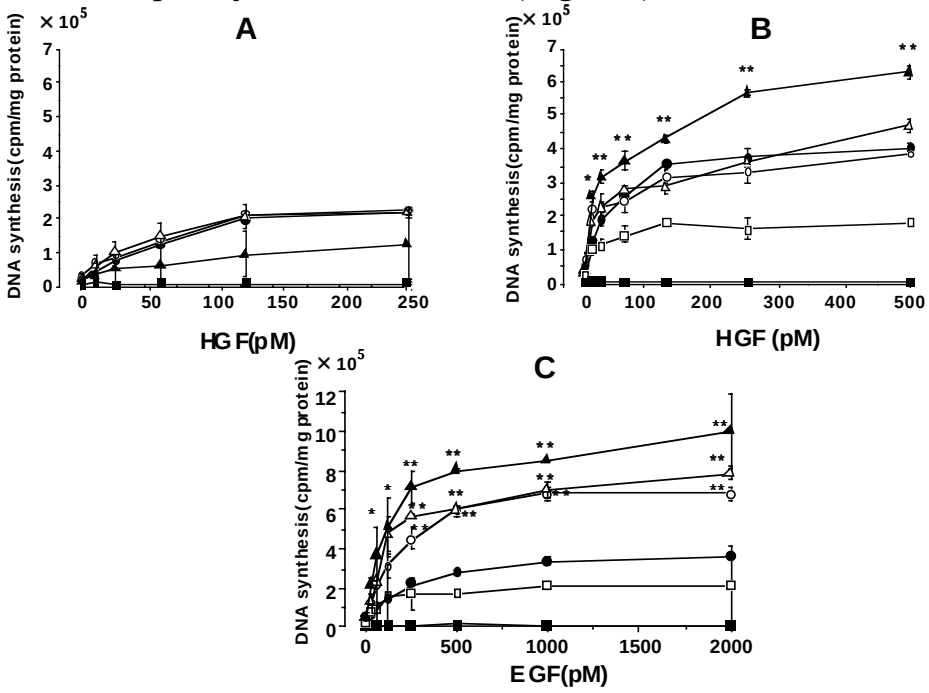

Fig. 6 Effect of protamine on the mitogenic response to HGF and EGF in primary cultured rat hepatocytes

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 (▲), 50 (□) and 200 (■) μg/ml. Ten minutes later, HGF (panel A, B) or EGF (panel C) was applied to give the indicated final concentrations, followed by the determination of DNA synthesis. Each value and vertical bar represent the mean ± S.E. of 3 rats.

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. 7). After intravenous administration of HGF (1 μg/kg), plasma HGF concentrations fell rapidly (Fig. 7). 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. 7). However, such a protamine dose-dependence differed from that for

the enhancing effect on the labeling index (Fig. 2) and reached a maximum at 20 mg/kg protamine (Fig. 7).

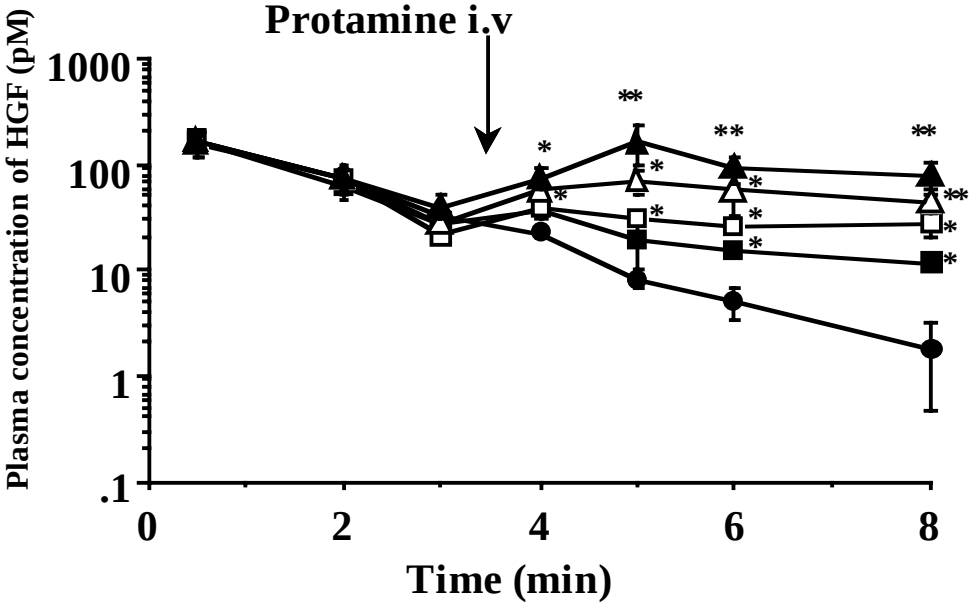

Fig. 7 Effect of protamine injection on the plasma elimination of HGF

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 the mean ± S.E. of 3 rats. * Significantly different from the control ($p < 0.05$); ** ($p < 0.01$).

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. 8A). The recovery of HGF from the heparin column was 85.7 % (Fig. 8A). 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. 8B). The HGF bound to the column was eluted by addition of protamine and the recovery of HGF was 84.4 % (Fig. 8B). After elution with protamine, only a small amount of HGF was further eluted by 2M sodium chloride (Fig. 8B). In this analysis we confirmed that the determination of HGF by EIA was not influenced by 20 mg/ml protamine (data not shown).

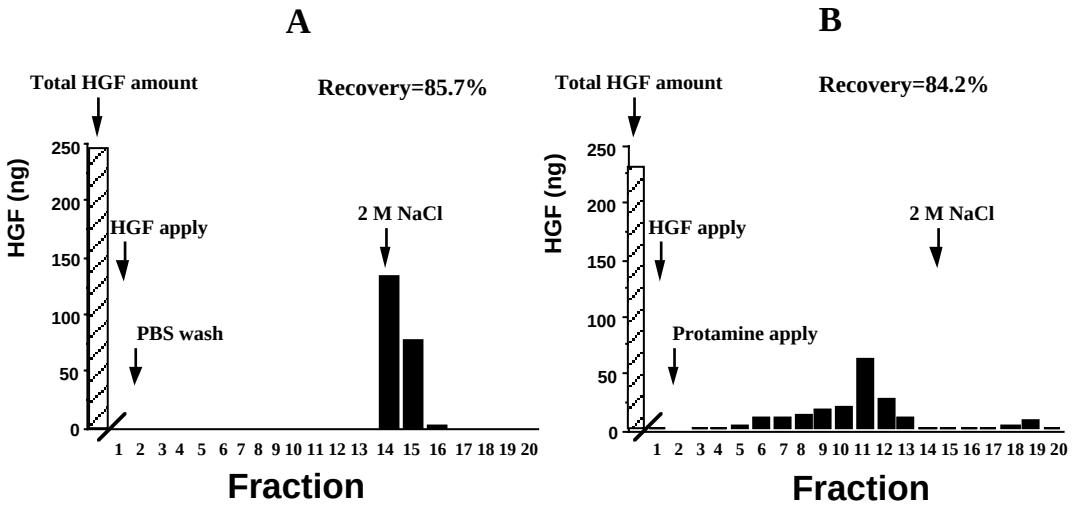

Fig. 8 Protamine elutes HGF prebound to a heparin-immobilized column

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.

DISCUSSION

In the present study, we found that protamine enhances HGF-induced liver regeneration when protamine is administered prior to injection of HGF (Fig. 1). Such an enhancing effect of protamine was found both in ANIT-intoxicated rats and partially hepatectomized rats (Fig. 2), and the protamine dose-dependence in the hepatocyte labeling indices was almost identical in both cases (Fig. 2A, 2B), suggesting that this effect may be general for a number of liver diseases. Protamine also significantly reduces γ-GTP further (Fig. 3) 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 (29). 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. 2). 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. 2A and Fig. 6) probably because of its cytotoxic effect.

The CL_{plasma} of HGF was reduced by preadministration of protamine (Fig. 4 and Table 1). 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 (2, 5). 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 (16-19). Considering that protamine has a high affinity for heparin (7) and can elute HGF molecules prebound to heparin-sepharose (Fig. 8), a transient increase in plasma HGF after intravenous administration of protamine (Fig. 7) 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. 4), the other is a direct stimulatory effect on the mitogenic response of hepatocytes to HGF (Fig. 6B). Protamine increases HGF AUC 2.5-fold (Table 1) 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. 1). 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. 5, HGF at a dose of 300 μg/kg plus protamine has a markedly higher labeling index than HGF alone at 500 μg/kg, but has an AUC nearly identical to that of HGF. The data shown in Fig. 5 provide clear evidence against a direct relationship between HGF availability (as expressed by the AUC) and liver regeneration (as expresssed by the labeling index). Thus, the mechanism of the effect of protamine on HGF-induced liver regeneration is not principally related to its inhibitory effect on HGF clearance. The difference in the protamine dose-dependence between the labeling index (Fig. 2) and plasma disappearance of HGF (Fig. 7), 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 in primary culture induced by HGF was increased approximately two-fold through the direct stimulatory effect of protamine (Fig. 6B). Therefore, we consider that the effect of protamine on the labeling index in vivo can also

be explained by considering such a direct effect of protamine on hepatocytes as one of the more rational mechanisms for the beneficial effects of protamine..

The effect of protamine on several cytokine receptors has been investigated (23, 28). Lokeshwar et al. (28) 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. 5C). This indicates that the direct enhancement effect of protamine on hepatocyte DNA synthesis is not specific to HGF. Sacks and McDonald (23) 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 (27). The diverse biological actions of HGF are a result of signaling through this receptor (22, 30). 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 (3). 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 significant (Fig. 3). 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, 1) a direct stimulatory effect on hepatocyte DNA synthesis and 2) indirect effect on HGF clearance which results in an increased exposure to HGF.

ACKNOWLEDGEMENTS

This study was supported in part by a Grant-in-Aid for Scientific Research provided by the Ministry of Education, Science and Culture of Japan. We are very grateful to Drs. Kohji Tanaka in Development Research Laboratories, Dainippon Pharmaceutical Co., LTD and Satoru Inagaki in the Development Research Laboratories, Banyu Pharmaceutical Co., LTD for kindly advising us how to determine the labeling index in hepatocytes in rats.

REFERENCES

1. Ando, T., M. Yamasaki, K. Suzuki. Protamines . In: Molecular Biology Biochemistry and Biophysics. A. Kleinzeller, G. F. Springer, and H. G. Wittmann (Eds.) Berlin. Springer-Verlag, 1973, Vol. 12: p. 1-30.
2. 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.
3. Heldin, C. H. Dimerization of cell surface receptors in signal transduction. Cell. 80: 213-223, 1995.
4. 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.
5. 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 (Renal Fluid Electrolyte Physiol. 34): F61-F69, 1993.
6. 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.
7. Jaques, L. B. Protamin-antagonist to heparin. J Can Med Assoc. 108: 1291-1297, 1973.
8. Kato, Y., K. X. 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.
9. Kato, Y., and Y. Sugiyama. Binding, internalization, degradation, and mitogenic effect of epidermal growth factor in cultured rat hepatocytes. STP Pharm. Sci. 3: 75-82, 1993.
10. 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.
11. Liu, K. X., 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 (Gastrointest. Liver Physiol. 26): G642-G649,

1992.
12. 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.
13. Liu, K. X., 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 (Gastrointest. Liver Physiol. 32): G745-G753, 1995.
14. Liu, K. X., Y. Kato, M. Yamazaki, O. Higuchi, T. Nakamura, and Y. Sugiyama. Decrease in the hepatic uptake clearance of hepatocyte growth factor (HGF) in CCl_4 -intoxicated rats. Hepatology. 17: 651-660, 1993.
15. Masumoto, A., N. Yamamoto. Sequestration of a hepatocyte growth factor in extracellular matrix in normal adult rat liver. Biochem. Biophys. Res. Commun. 174: 90-95, 1991.
16. 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.
17. 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.
18. 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.
19. 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.
20. 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 (Lung cell. Mol. Physiol. 14): L1031-L1039, 1996.
21. O'Reilly, R. A. In: Anticoagulant, antithrombotic, and thrombolytic drugs. The pharmalogical Basis of Therapeutics. A.G. Goodman., L. S. Gilman, and A. Gilman(Eds.) New York. Macmillan. 1980, p. 1347-1366.
22. 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.
23. 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.
24. 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.
25. 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.
26. 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.
27. 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.
28. 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.
29. 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.
30. 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.
31. 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.
32. Zarnegar, R., M. C. DeFrances, L. Oliver, and G. K. Michalopoulos. Identification and partial characterization of receptor binding sites for HGF on rat hepatocytes. Biochem. Biophys. Res. Commun. 173: 1179-1185, 1990.