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RUNNING HEAD

Enhancing Effect of Protamine on Activity of HGF

Abstract

Objective. The aim of the present study was to characterize the mechanism of the stimulatory effect of protamine on HGF activity.

Methods. The enhancing effects of protamine on the proliferative activity of HGF were investigated *in vivo,* in primary cultured rat hepatocytes, and in perfused rat liver.

Results. In α-naphthylisothiocyanate-intoxicated rats, pretreatment with protamine increased HGF-induced autophosphorylation of the HGF receptor in liver. The maximum enhancing effect of protamine on HGF-induced DNA synthesis of hepatocytes required a 10 minute-pretreatment period both *in vivo* and *in vitro*, and the stimulatory effect of protamine was not observed when it was administered simultaneously with HGF. Preperfusion of the liver with protamine for 10 minutes decreased the non-saturable portion of hepatic clearance for ^{125}I-HGF, which is mainly mediated by cell-surface heparan-sulfate proteoglycan (HSPG). Inhibition of HGF binding to heparin by protamine was confirmed using heparin-coated sepharose. This inhibition also required 10 minutes of pretreatment, for protamine to bind heparin.

Conclusion. The enhancing effect of protamine on the mitogenic activity of HGF on hepatocytes requires pretreatment with protamine for a short period presumably required for its binding to cell-surface heparin, implying possible regulation of c-met autophosphorylation by HSPG.

KEY WORDS: Hepatocyte growth factor (HGF); Protamine; Heparin; Heparan-sulfate proteoglycan (HSPG)；Clearance.

INTRODUCTION

Hepatocyte growth factor (HGF), is a biologically active polypeptide with a molecular weight of 82-85 kD, composed of a 69 kD α-subunit and a 34 kD β-subunit (1, 2). It is a potent mitogen for mature parenchymal hepatocytes (3, 4). In addition, HGF has other activities such as stimulation of cell motility (5), inhibition of tumor cell growth (6) and proliferation of many types of epithelial cells (7-9). In hepatic malfunctions such as partial hepatectomy or CCL_4-induced hepatitis, HGF activity and HGF mRNA dramatically increase in both plasma and liver (10-12). HGF is therefore considered to act as a hepatotropic factor in the repair of injured liver.

The biological activity of HGF is exerted through its binding to a specific receptor, a transmembrane tyrosine kinase that is the product of the protooncogene c-met (13,14). The HGF receptor consists of an extracellular, 50 kD α chain, and a 145 kD β chain that has extracellular, transmembrane and intracellular regions (14). The intracellular domain of the ß subunit contains a tyrosine kinase domain and sites for autophosphorylation through which the cellular effects of HGF receptor are mediated. HGF binding to the receptor has been suggested to induce receptor dimerization, resulting in reciprocal trans-phosphorylation of each receptor and subsequent interaction with the other cytoplasmic effectors, through which it exerts its biological effects (15, 16).

Although HGF is expected to be clinically useful for repairing tissue injury, its systemic administration may be hindered by its rapid elimination from circulation (10, 11, 17, 18), which leads to a requirement for large amounts of repeated doses to obtain pharmacological activity (19). Therefore, it is essential to develop a drug delivery system (DDS) that increases the efficacy of HGF mitogenic activity *in vivo*. HGF binds to heparan-sulfate proteoglycan (HSPG), which is expressed ubiquitously on the surface of cells, and in the extracellular matrix (20, 21). Systemic elimination of HGF is mediated at least partially via these heparin-like substances, which are primarily expressed in the liver (17, 20). Therefore, inhibition of this

binding may lead to stability of HGF in circulation. Recently, it was found that mutational deletion of the heparan sulfate binding site in HGF increases the biological activity of HGF (22, 23), supporting the validity of this strategy.

Finding an effective DDS for HGF may promote clinical applications of HGF. We have previously demonstrated that protamine, a basic protein with an affinity for heparin, not only decreases the overall elimination of HGF, but also enhances the proliferative activity of HGF in the liver of α-naphthylisothiocyanate (ANIT)-intoxicated rats (19). Based on these previous findings, coadministration of HGF with protamine may be a useful DDS for HGF that increases pharmacological efficacy *in vivo*. However, the molecular mechanism by which protamine enhances HGF-induced liver regeneration is still unclear. Protamine has been widely used as a carrier for gene delivery because of its potential affinity for nucleotides. Cytotoxicity exerted by polycationic polypeptides like protamine may be one of the stumbling blocks in the development of DDS. On the other hand, the use of protamine for the delivery of HGF or other heparin-binding proteins has not yet been reported except our approach (19).

The aim of the present study was to characterize the enhancing effect of protamine on the proliferative activity of HGF. The present study revealed that protamine affects the early-phase of signal transduction, possibly through its activity of binding to cell-surface HSPG.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 240-250 g (Nisseizai, Tokyo, Japan) were used and treated in accordance with guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan). ANIT dissolved in olive oil was intraperitoneally injected at a dose of 50 mg/kg body wt.

Materials

Protamine sulfate purified from salmon roe was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); ANIT, 3,3'-diaminobenzidine, Azure A and heparin 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 a plasmid containing human HGF cDNA (24). Mouse monoclonal antibodies to phosphotyrosine (p-Tyr, PY20) and rabbit polyclonal antibodies to HGF receptor [m-met (SP260)] were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [^{125}I]Anti-rabbit IgG was from GE Healthcare Biosciences (Buckinghamshire, UK).

HGF and Protamine Injection

Under light ether anesthesia, HGF (300 mg/kg body wt) dissolved in saline was administered through the penis vein 30 min before and 8, 22, 32, 46 h after ANIT intoxication. Protamine (1.6 mg/kg body wt) dissolved in saline was administered 10, 30 and 120 min prior to the HGF injection or simultaneously administered with HGF via the same route. In our previous analysis (19), the peak value of the labeling index occurred at a protamine dose of 1.6 mg/kg, and the higher dose of protamine decreased the labeling index induced by HGF in ANIT-intoxicated rats. Therefore, we measured the labeling index only at 1.6 mg/kg of protamine in the present study. Rats were sacrificed, and livers were excised 48 h after ANIT intoxication for the determination of labeling index and Western blot analysis.

Measurement of Labeling Index

One hour before sacrifice, 5-bromo-2'-deoxyuridine dissolved in normal saline was intraperitoneally injected at 100 mg/kg body wt. Rats were exsanguinated via the abdominal artery under light ether anesthesia. The liver was removed and fixed in 10% buffered formalin for 24 h. The fixed samples were embedded in paraffin, and 4 μm paraffin sections were mounted on glass slides. 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 (GE Healthcare Biosciences). The labeling index of hepatocytes was determined by counting more than 500 nuclei from photographs of three randomly selected light microscopy fields.

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 24 h as described previously (4). Non-attached cells were removed by washing, and culture medium containing protamine applied to the monolayer. HGF or EGF was added 10 sec, 10 min, 30 min, 1 h and 3 h after the addition of protamine, or added simultaneously with protamine. ^{125}I-deoxyuridine was added 22 h after HGF addition, and its incorporation after 6 h was assayed as described previously (4). Cellular protein was determined by the Bradford method using Bio-Rad protein assay kits with bovine serum albumin (BSA) as a standard.

Determination of HGF Concentration in Primary Cultured Rat Hepatocyte Medium

After 24 hr of growth, HGF was added to primary cultures, either alone or 10 min after addition of 25 μg/ml of protamine. At 0, 1, 3, 6 and 24 h after HGF addition, 50 μl of medium was collected, and the HGF concentration (C_m) was determined by ELISA (Institute of Immunology Co., LTD., Tokyo, Japan). The time profile for C_m was fitted to a mono-exponential equation by a nonlinear iterative least squares method (17). The input data were weighted as the reciprocal of the square of observed values using the Damping Gauss Newton Method as the algorithm for fitting. The HGF clearance was calculated by dividing the dose applied to the medium by the area under the concentration-time curve (AUC). The AUC was obtained by integration of the mono-exponential equation from time 0 to infinite.

Liver Perfusion Experiment

Liver perfusion was as previously reported (11,17). In brief, 24 h after ANIT intoxication, single-pass perfusion was performed from rat portal veins with oxygenated perfusion buffer containing 3% (w/v) BSA at a perfusion rate of 30 ml/min in a temperature-controlled cabinet at 37°C. ^{125}I-HGF (1 μCi/ml) or ^{14}C-taurocholate (TCA, 1 μCi/ml) with ^{14}C or ^{3}H-inulin (5 μCi/ml) was injected into the portal vein as a bolus at indicated times after preperfusion with protamine for the indicated periods. After 30 s, the liver was excised, and radioactivity remaining in the liver was determined. The hepatic extraction ratio (E) of ^{125}I-HGF or ^{14}C-TCA was calculated as follows:

$$E_{HGF} = E_{app,HGF} - E_{app,inulin} \quad (1)$$

$$E_{TCA} = E_{app,TCA} - E_{app,inulin} \quad (2)$$

where $E_{app,HGF}$, $E_{app,TCA}$ and $E_{app,inulin}$ were the apparent extraction ratio for ^{125}I-HGF, ^{14}C-TCA and ^{14}C-Inulin, respectively, determined by total radioactivity associated with the liver divided by the radioactivity injected into the portal vein.

Determination of Protamine in Plasma

Under light ether anesthesia, protamine (0 or 1.6 mg/kg) was administered through the penis vein 24 h after ANIT intoxication. Plasma was collected from the external jugular vein, and the protamine concentration determined using the Azure A-heparin complex method (25). Briefly, 400 μl of the plasma sample was added to 600 μl of assay solution containing 15 mg/ml of Azure A dye and 3 unit/ml of heparin dissolved in distilled water. The absorbance was then measured at 620 nm against the reference sample containing the same concentration of Azure A dye and heparin but no protamine.

The plasma concentration (C_p) - time profiles of protamine after intravenous administration were fitted to the following two-exponential equation

$$C_m = A \exp(-\alpha t) + B \exp(-\beta t) \quad (3)$$

where α and β are the apparent rate constants, A and B are the corresponding zero-time intercepts, and t is time. The AUC was calculated as:

$$AUC = A/\alpha + B/\beta \quad (4)$$

The half-life for β-phase was calculated as (ln 2)/β. The plasma clearance (CL_{plasma}) and initial plasma concentration (C_0) were calculated as Dose/AUC and the sum of A and B, respectively.

Autophosphorylation of HGF Receptor

At 48 h post-ANIT-intoxication, the liver was excised, homogenized and solubilized with lysis buffer (PBS containing 1% Triton X-100). The lysate (800 mg protein) was added to protein A-sepharose to remove non-specific binding, and the supernatant incubated with anti-HGF receptor antibody at 4 ^{o}C for 2 h, followed by further incubated with protein A-sepharose beads. The beads were washed three times with lysis buffer and immunoprecipitates boiled in Laemmli sample buffer, separated by 7.5% polyacrylamide-SDS gel electrophoresis and transferred to PVDF membranes (Millipore Corp., Billerica, MA). Membranes were blocked in Tris-buffered saline containing 5% BSA at 4°C overnight, and then incubated with anti-HGF receptor antibody or anti-phosphotyrosine monoclonal antibody for 3 h. After washing, membranes were incubated with ^{125}I-anti-rabbit IgG for 1 h, and the densities of HGF receptor in phosphorylated or unphosphorylated form were detected by imaging analyzer (BAS III, Fujifilm, Tokyo, Japan).

Binding of Protamine and HGF to Heparin Sepharose Beads

To analyze the binding of protamine to heparin, 20 µl of heparin sepharose bead solution (heparin sepharose beads:PBS at 1:1) was incubated at 37°C with protamine at a final concentration of 0.025 - 1.0 µg/ml. The beads were washed with ice-cold PBS, and collected by centrifugation. Protamine bound to heparin sepharose beads was eluted with 20 µl of NaCl (1 M), and the concentration determined by the Azure A-heparin complex method (25) as

described above. Scatchard analysis was also performed to obtain dissociation constant (K_d) and maximum binding capacity (B_{max}).

To analyze the binding of HGF to heparin, 40 µl of heparin sepharose beads were incubated with 10 µl of HGF at a final concentration of 100-1000 µg/ml. The beads were washed with ice-cold PBS, and collected by centrifugation. Bound HGF was eluted with 40 µl of 2 M NaCl, and HGF concentration determined by ELISA.

Statistical Analysis

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

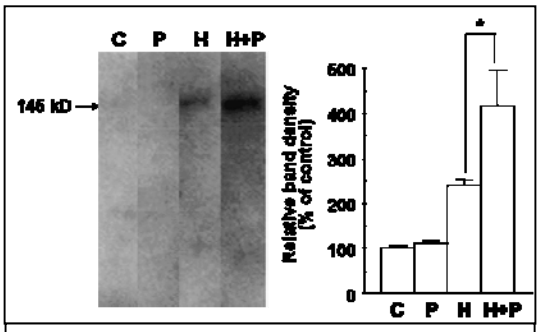

Fig. 1 Effect of protamine on HGF-induced phosphorylation of HGF receptor *in vivo*

RESULTS

Effect of Protamine on HGF-Induced Phosphorylation of the HGF Receptor

To characterize the enhancing effect of protamine on the proliferative activity of HGF, we first focused on phosphorylation of the HGF receptor, which is the earliest event in the signal transduction pathway. As per our previous *in vivo* analysis (19), protamine (1.6 mg/kg) was preinjected 10 min prior to injection of HGF. In this study, phosphorylation of the HGF receptor was examined 48 h after ANIT-intoxication. Receptor phosphorylation after injection of HGF alone (300 µg/kg) was found to be 2.7-fold higher than the control (Fig. 1). Pretreatment with protamine further increased HGF receptor phosphorylation by an additional 1.5-fold, and protamine alone did not affect HGF receptor phosphorylation (Fig. 1).

Effect of Protamine Pretreatment Time on HGF-Induced Proliferation *In Vivo*

To determine the most suitable preinjection period for protamine, the mitogenic effect of

HGF was examined in ANIT-intoxicated rats at various protamine preinjection times (Fig. 2). The labeling index of ANIT-intoxicated rat hepatocytes after administration of protamine 10 min prior to HGF injection was 3.3-fold than that with injection of HGF alone (Fig. 2). However, when protamine was simultaneously injected, or injected 30 or 120 min prior to HGF injection, the labeling index was nearly comparable to HGF alone (Fig. 2). Thus, the mitogenic effect of HGF was greatest after protamine pretreatment for 10 min prior to HGF injection.

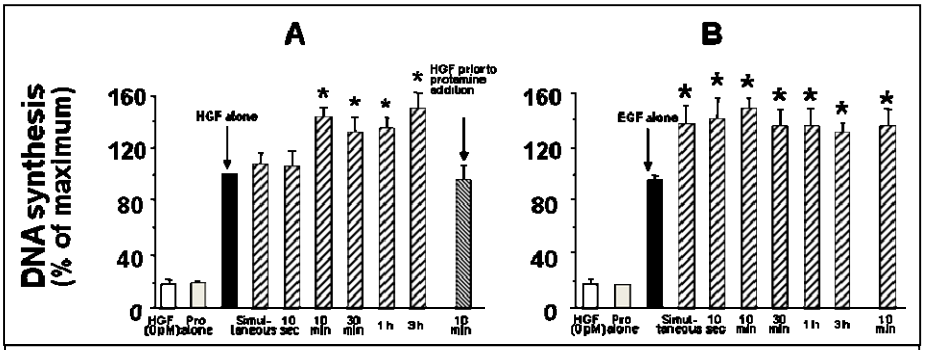

Fig. 3 Effect of protamine pretreatment period on the mitogenic effect of HGF (A) and EGF (B) in primary cultured rat hepatocytes

Effect of Protamine Pretreatment Time on HGF-Induced DNA Synthesis of Hepatocytes *In Vitro*

To further study the effect of pretreatment with protamine on HGF proliferative activity, DNA synthesis of HGF-treated hepatocytes in primary culture was examined after various protoamine pretreatment times (Fig. 3). Protamine was used at 25 μg/ml, since this concentration exerted the maximum stimulatory effect on HGF-induced DNA synthesis of hepatocytes in primary culture (19). When protamine was simultaneously added with HGF (250 pM), or added 10 sec prior to HGF addition, DNA synthesis was nearly the same as with HGF alone (Fig. 3A). However, DNA synthesis increased to 1.3 - 1.5-fold over the control level when protamine was

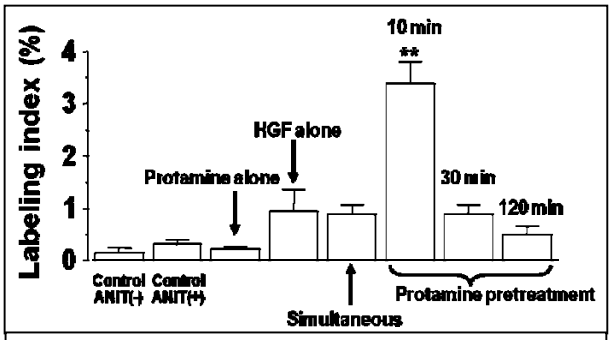

Fig. 2 Effect of protamine pretreatment period on HGF-induced liver regeneration in ANIT-intoxicated rats

added at 10 or 30 min, or 1 or 3 h prior to HGF addition (Fig. 3A). When the same concentration of HGF was added 10 min prior to protamine addition, however, DNA synthesis was approximately equal to that in the presence of HGF alone (Fig. 3A). Similar results were also observed at a lower concentration of HGF (25 pM, data not shown).

Similar studies were also performed for EGF, another hepatocyte mitogen, since protamine also enhanced EGF-induced stimulation of primary hepatocyte DNA synthesis (19). In this case, DNA synthesis in the presence of EGF (500 pM) was higher after any protamine pretreatment time (Fig. 3B). Similar results were observed at a lower EGF concentration (60 pM, data not shown).

Rapid *In Vivo* Disappearance of Protamine in Systemic Circulation

Pretreatment with protamine for 30 min or longer increased the *in vitro*, but not the *in vivo* proliferative effect of HGF (Fig. 3A). One possible reason for this discrepancy could be rapid systemic elimination of protamine, so pharmacokinetic characterization of protamine was examined. The plasma concentration of protamine rapidly disappeared with a $t_{1/2,}$ and CL_{plasma} of 9 min and 5.3 ml/min/kg, respectively, after intravenous injection (Fig. 4). The C_0 was estimated to be 24.6 ± 1.0 μg/ml, which coincided with the effective concentration of protamine that stimulated HGF-induced DNA synthesis of hepatocytes *in vitro* (19). The plasma protamine concentration decreased to 10.2 ± 0.2 μg/ml at 10 min after injection (Fig. 4). This concentration of protamine could not enhance HGF-induced DNA synthesis in primary cultured hepatocytes (19).

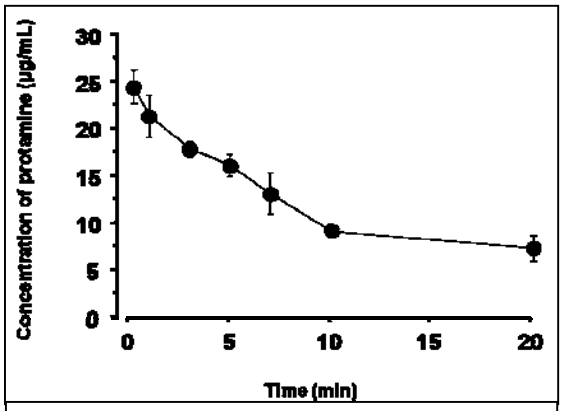

Fig. 4 Elimination profile of protamine after intravenous administration in rats

Effect of Protamine Pretreatment on Low-Affinity Clearance Sites for ^{125}I-HGF in

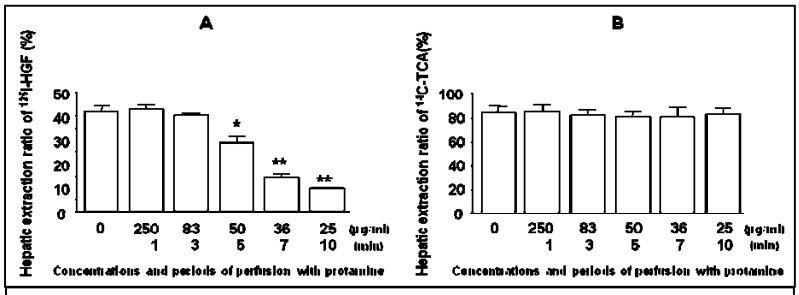

Fig. 5 Effect of protamine preperfusion period on hepatic extraction ratio of ^{125}I-HGF (A) and ^{14}C-TCA (B)

Perfused Rat Liver

To further characterize the effect of protamine pretreatment on hepatic processing of HGF, we determined the change in hepatic clearance of HGF, assessed as E_{HGF} of ^{125}I-HGF in perfused rat liver, at varying pretreatment times with a constant amount of perfused protamine (Fig. 5A). With 1 or 3 min pretreatment periods, the E_{HGF} of ^{125}I-HGF was nearly comparable with the control (Fig. 5A). However, when the pretreatment period was increased to 5, 7 or 10 min, the E_{HGF} value decreased in a manner dependent on the pretreatment period (Fig. 5A). The E_{TCA} was almost unchanged with protamine pretreatment under the same conditions (Fig. 5B). Pretreatment with protamine at 25, 36, 50 and 83 μg/ml for 1 min gave an E_{HGF} almost equal to the control (data not shown).

Association of HGF on hepatocytes is mediated by high-affinity and low-affinity clearance sites, which are presumably mediated by the HGF receptor and a heparin-like substance, respectively (17). To examine the effect of protamine pretreatment on these two sites separately, E_{HGF} was measured in the presence of excess unlabeled HGF (500 pM), sufficient to saturate the high-affinity site (17) (Table 1). The E_{HGF} value in the absence of unlabeled HGF decreased after 10 min of pretreatment with protamine (25 μg/ml) (Table 1). A similar effect for protamine preperfusion was observed for the E_{HGF} in the presence of unlabeled HGF (500 pM), but was not seen for the specific extraction of ^{125}I-HGF obtained by subtracting E_{HGF} in the presence of unlabeled HGF from E_{HGF} in its absence (Table 1). Thus, the effect of protamine pretreatment was more apparent on association of HGF with the low-affinity binding site.

Table 1. Effect of Protamine on Hepatic Extraction of ^{125}I-HGF in Perfused Rat Liver[a)]

HGF concentration	Protamine concentration (μg/ml)	E_{HGF}[b)] % (Mean ± SEM)	E_{TCA}[b)] % (Mean ± SEM)
Tracer ^{125}I-HGF alone	0	43.7±1.0	89.6 ± 1. 1
	25	30.9±1.4	87.0 ± 3.3
Tracer ^{125}I-HGF + 500 pM HGF[c)]	0	14.9±0.6	88.6 ± 3.1
	25	6.40±0.3	85.4 ± 3.6
Specific extraction[d)]	0	28.8±1.5	
	25	24.5±1.1	

a) During a single-pass perfusion of ANIT-intoxicated rat liver, ^{125}I-HGF or ^{14}C-TCA was injected into the portal vein just after preperfusion with protamine for 10 min, followed by determination of radioactivity remaining in the liver 30 sec later.

b) The hepatic extraction ratio of ^{125}I-HGF and ^{14}C-TCA was calculated by Eqs. (1) and (2), respectively.

c) Unlabeled HGF (500 pM) was co-perfused for 1 min before the radioactivity determination.

d) Specific extraction was obtained by subtracting the E_{HGF} of ^{125}I-HGF in the presence of unlabeled HGF, from the E_{HGF} in its absence.

Effect of Protamine on Binding of HGF to Heparin

The effect of protamine on the association of HGF with the low-affinity binding site (Table

1) may imply an inhibitory effect of protamine on HGF binding with HSPG on the liver cell-surface. To investigate the effect of protamine on HGF binding to heparin, the binding characteristics of protamine itself to heparin (Fig. 6A, 6B), and the effect of protamine on HGF binding to heparin (Fig. 6C), were examined using heparin sepharose beads. The amount of protamine binding to heparin sepharose beads increased in a time-dependent manner from 0.25 to 10 min, with maximum binding observed at 10 min (Fig. 6A). After 10 min, the binding of protamine plateaued (Fig. 6A). Binding of protamine to heparin was further characterized by Scatchard plot analysis (Fig. 6B). The K_d and B_{max} were 1.00 μM and 24.0 nmol/ml beads, respectively (Fig. 6B).

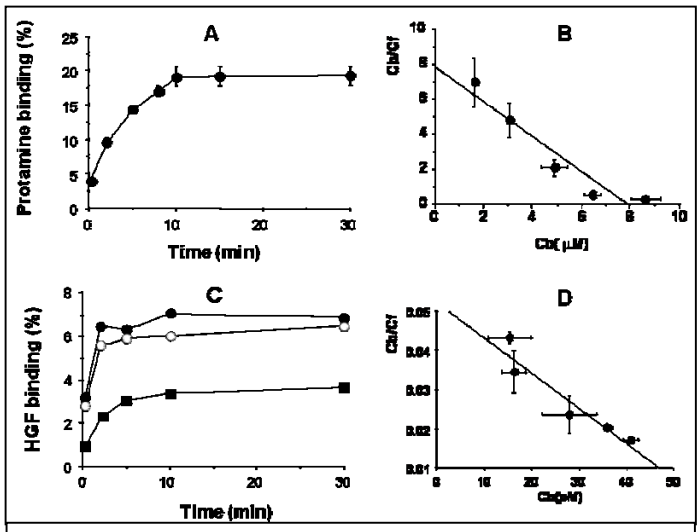

Fig. 6 Binding of protamine (A, B) or HGF (D) to heparin and inhibition by protamine of HGF binding to heparin (C)

HGF also binds to heparin sepharose beads, and binding reached maxmimal levels within 2 min (Fig. 6C). A similar binding pattern was also found when both HGF and protamine were simultaneously added to heparin sepharose beads (Fig. 6C),but when protamine was added 10 min prior to HGF addition, the binding of HGF decreased to 30 - 50% of the control level, and the binding reached a plateau within 10 min (Fig. 6C). Binding of HGF to heparin was further characterized by Scatchard plot analysis, yielding K_d and B_{max} values of 1.11 nM and 0.145 pmol/ml beads, respectively (Fig. 6D).

Protamine increases the maximum HGF-induction of primary hepatocyte proliferation

The enhancing effect of protamine on HGF-induced DNA synthesis of hepatocytes (19) might be explained at least in part by the inhibition of hepatocellular clearance of HGF by protamine. In the present study, to separately estimate the effect of protamine on the mitogenic effect and the clearance of HGF, both the DNA synthesis of hepatocytes in primary culture and the HGF concentration profile in the medium were measured (Fig. 7). HGF in the medium gradually disappeared, and protamine (25 μg/ml) hindered this disappearance (Fig. 7A). The HGF clearances were 23.0 ± 4.6 and 5.81 ± 2.30 nl/min/mg protein in the absence and presence of protamine, respectively (Fig. 7A). These values corresponded to 27.7 and 15.1 ml/min/kg body wt, if we assume that 1 g liver corresponds to 118 mg protein, 1 kg body wt includes 40 g liver, and the hepatic plasma flow rate equals 36.4 ml/min/kg body wt (26, 27). These extrapolated values were close to the observed values for plasma clearance of HGF (32.1 and 12.7 ml/min/kg in the absence and presence of protamine, respectively) (19). DNA synthesis in the presence of HGF, with or without protamine pretreatment, was then measured and plotted against AUC of HGF in medium, to avoid the effect of protamine on HGF clearance (Fig. 7B). In the absence of protamine, DNA synthesis reached nearly the maximum value at 250 pM of HGF (Fig. 7B). In the presence of protamine, DNA synthesis with 25 - 1,000 pM of HGF was higher than that in the absence of protamine (Fig. 7B). The enhancing effect of protamine was more apparent for DNA synthesis induced by higher concentrations of HGF (250 - 1,000 pM), whereas the protamine effect on DNA synthesis induced by 25 pM HGF could be almost fully explained by the inhibition of HGF clearance, since DNA synthesis at 25 pM HGF with protamine was superimposable on the line obtained in the absence of HGF (Fig.7B).

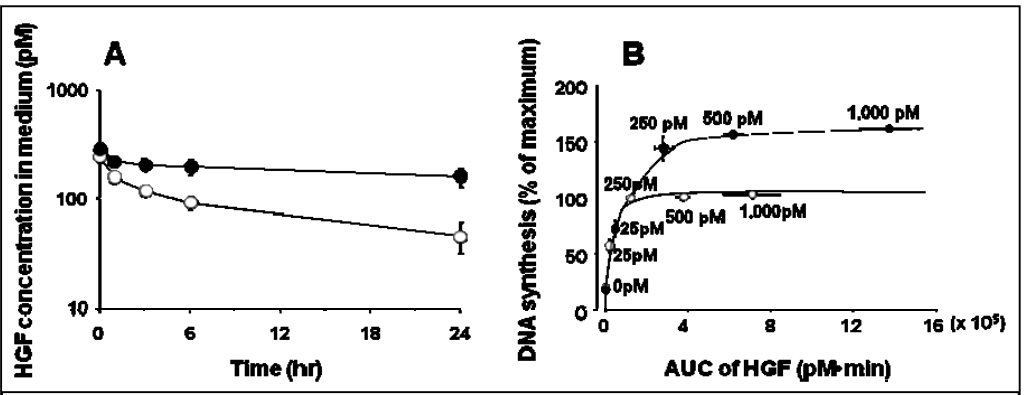

Fig. 7 Effect of protamine on HGF elimination and HGF-induced DNA synthesis in rat hepatocytes

DISCUSSION

HGF exerts mitogenic, morphogenic and motogenic activities in various types of cells (28, 29). These physiological activities are all initially mediated by c-met tyrosine kinase, the receptor for HGF (13). In the present study we found that protamine preinjection significantly enhanced the effect of HGF on phosphorylation of the HGF receptor, and protamine alone did not stimulate phosphorylation (Fig.1). Protamine pretreatment did not enhance the amount of HGF receptor, according to Western blot analysis using anti-HGF receptor antibody (data not shown). These results suggest that the effect of protamine could be relevant to the early-phase event in signal transduction from the HGF receptor. We have previously reported that protamine enhances the proliferative activity of HGF on hepatocytes (19). This enhancement could be explained at least in part by protamine stimulation of HGF-induced receptor phosphorylation (Fig. 1).

Since the effect of protamine is relevant to this early-phase event, we determined the length of protamine pretreatment that was required to stimulate the proliferative activity of HGF. Protamine pretreatment periods from 0 to 120 min were tested (Fig. 2). *In vivo,* the enhancing effect of protamine on HGF-induced liver regeneration depended strongly on the protamine pretreatment period (Fig. 2). The pretreatment period with protamine for 10 min seems to be essential for the enhancing effect of protamine on the proliferative activity of HGF (Fig. 2). This was, in principle, consistent with the observations of HGF-induced DNA synthesis in primary cultured hepatocytes, although the protamine effect in primary cultures was maintained for over 30 min prior to HGF addition (Fig. 3A).

The unique characteristics of the protamine effect prompt questions about the possible reasons for such a strict preincubation period. Some answers are provided by the profile of plasma protamine concentration over time, after intravenous injection into ANIT-intoxicated rats. The C_0 of protamine was found to be 25 μg/ml, falling to 10 μg/ml just 10 min after intravenous

injection (Fig. 4). According to our previous analysis with primary cultured rat hepatocytes, HGF-induced DNA synthesis was enhanced by 25 μg/ml, but not by 6 - 12.5 μg/ml of protamine (19). Thus, effective protamine concentration in circulating plasma for enhancing HGF activity was maintained for only approximately 10 min after intravenous injection *in vivo* (Fig. 4). This could be the reason for the minimal enhancement of protamine on HGF activity *in vivo* when the pretreatment period was 30 min or longer (Fig. 1). The protamine concentration in cultured hepatocyte medium could be maintained for a longer period than *in vivo*, explaining why enhancing effects of protamine were observed with pretreatment periods of 30 min or longer (Fig. 3A).

The strict period of approximately 10 min of protamine pretreatment may also be relevant to the time required for protamine to bind to heparin. Protamine is a basic protein and binds to heparin (30). In the present study, the preperfusion effect of protamine was also observed on E_{HGF} (Fig. 5A) and more pronounced when E_{HGF} was measured in the presence of excess unlabeled HGF (Table 1), suggesting that protamine mainly inhibits low-affinity HGF binding on the liver cell-surface. Since the high- and low-affinity binding sites for HGF on liver cells mainly represent HGF receptor and HSPG, respectively (17), protamine mainly inhibits the binding of HGF to HSPG. In fact, we demonstrated that protamine binds to heparin (Figs. 6A, 6B) and inhibits the binding of HGF to heparin (Fig. 6C). Interestingly, approximately 10 minutes was necessary for maximum binding of protamine to heparin (Fig. 6A). This coincided with the time required for the maximum stimulatory effect of protamine on HGF proliferative effects *in vivo* (Fig. 2) and *in vitro* (Fig. 3), and the maximum inhibitory effect of protamine on hepatic extraction of HGF (Fig. 5A). In addition, simultaneous addition of protamine with HGF minimally affected the binding of HGF to heparin (Fig. 6C), and consistent with the finding that simultaneous addition of protamine with HGF did not increase the proliferative activity of HGF *in vivo* (Fig. 2) or *in vitro* (Fig. 3). According to these results, 10 min may be required for protamine to occupy HSPG on cell-surfaces and inhibit HGF binding. On the other hand, HGF

is also a basic protein and has an affinity for heparin (31, 32). HGF can bind to heparin sulfate expressed on the surface of ubiquitous cells and in the extracellular matrix (20, 21). In the present study HGF bound to heparin more rapidly than protamine (Figs. 6A, 6C). Compared with HGF, protamine had a much lower affinity for heparin, with a 1000-fold difference in Kd between the two proteins (Fig. 6B, 6D). This may explain why the enhancing effect of protamine was almost completely abolished when protamine and HGF were simultaneously injected or added (Figs. 2, 3A).

We previously proposed that the combination of HGF and protamine may provide an adequate DDS for HGF, enhancing the proliferative activity of HGF and decreasing its systemic clearance (19). Although HGF is expected to be clinically useful for repairing tissue injury, rapid elimination of HGF from circulation may hinder clinical applications. Binding HSPG with protamine may be a rational strategy for inhibiting the HSPG-mediated HGF elimination pathway (19). However, considering the present findings, strict control over the timing of sequential administration of protamine and HGF may be required for optimal pharmacological efficacy. In addition, the labeling index occurred at a protamine dose higher than 1.6 mg/kg was decreased in ANIT-intoxicated rats *in vivo*, and HGF-induced DNA synthesis in primary cultured hepatocytes *in vitro* was decreased in the presence of 50-200 μg/ml of protamine, both of these findings being probably due to the cytotoxicity exerted by protamine (19). Existence of such optimal dose and/or concentration of protamine may also complicate the application of protamine to DDS. Alternatively, it may be important to find proteins other than protamine that have a much higher affinity for heparin, and can more strongly stimulate the proliferative activity of HGF.

In the present study, we found that protamine delayed the elimination of HGF from the medium of primary cultured hepatocytes (Fig. 7A, 7B), confirming the previous *in vivo* finding (19). At 25 pM HGF, the enhancing effect of protamine on HGF-induced DNA synthesis might be explained by the increase in AUC of HGF, since the data for DNA synthesis in the presence

of protamine was almost superimposable on the data for HGF alone (Fig. 7B). The mitogenic effect of HGF alone reached maximum value at 250 pM HGF (Fig. 7B). On the other hand, when protamine was added prior to HGF, the maximum DNA synthesis increased further, and this could not be explained by an increase in AUC (Fig. 7B). Thus, the stimulating effect of protamine was much more apparent at higher HGF concentrations (>250 pM), in which the HGF receptor is completely occupied, according to the 20 - 40 pM dissociation constant of HGF to HGF receptor. On the other hand, preperfusion with protamine mainly affected the low-affinity site for HGF (Table 1), suggesting that protamine mainly interacts with heparin. Naka et al. (33) and Schwall et al. (34) proposed that the mitogenic activity of HGF on hepatocytes is regulated by HSPG. Protamine might first bind to cell-surface heparin, affecting signal transduction between heparin and the HGF receptor at the cell-surface, and leading to stimulation of HGF receptor autophosphorylation (Fig. 1). Several recent studies have shown that, upon tyrosine kinase phosphorylation, the HGF receptor is associated with a number of SH2-containing signal mediators, such as the Ras GTPase-activation protein, the p85 subunit of phosphatidylinositol (PI) 3-kinase, phospholipase C (PLC) γ1, and a cytoplasmic tyrosine kinase of the Src family (35-37). PLCγ1 is also reported to mediate an intracellular signal for the HGF-enhanced mitogenesis of primary cultured rat hepatocytes (38). Whether the enhancing effect of protamine on HGF-induced liver regeneration is also related to these mediators remains to be elucidated.

EGF is another hepatocyte mitogen (39). Until now, an affinity of EGF for heparin has not been found. Lokeshwar et al. (40) reported that, in Swiss 3T3 cells and human epidermoid carcinoma A431, protamine induced an increase in the number of EGF receptors, which are also transmembrane tyrosine protein kinase receptors, by activating cryptic or inactive receptors to functionality. This may be relevant to our finding that the enhancing effect of protamine on EGF-induced DNA synthesis was not dependent on the timing of addition of protamine (Fig. 3B).

CONCLUSION

Protamine enhances HGF-induced phosphorylation of the HGF receptor, possibly by inhibiting the binding of HGF to a heparin-binding substance on the cell-surface. The protamine effect requires approximately 10 min of pretreatment, because of its relatively low affinity for heparin.

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Figure Legends

Figure 1. Effect of protamine on HGF-induced phosphorylation of HGF receptor *in vivo*.

Saline (C), protamine (1.6 mg/kg) (P), HGF (300 μg/kg) (H) or HGF (300 μg/kg) with protamine (1.6 mg/kg at 10 min prior to HGF injection) (H+P) was intravenously administered in ANIT-intoxicated rats, and livers homogenized 48 h after ANIT-intoxication. Phosphorylation of the HGF receptor was detected by immunoprecipitation with anti-HGF receptor antibody and subsequent western blot analysis using anti-phosporylated tyrosine antibody. The left panel represents a typical western blot, with relative band densities (% of control) shown in the right panel, in which each value and vertical bar represents the mean ± SEM of three determinations.

*: Significantly different from HGF alone ($p < 0.05$).

Figure 2. Effect of protamine pretreatment period on HGF-induced liver regeneration in ANIT-intoxicated rats.

In ANIT-intoxicated rats, protamine (1.6 mg/kg) was intraveously administered alone, or at the indicated minutes prior to injection of HGF (300 μg/kg). The labeling index of hepatocytes was determined 48 h after ANIT intoxication. ANIT(+) and ANIT(-) represent ANIT-intoxicated rats, and non-intoxicated rats treated with saline. Each value and vertical bar represents the mean ± SEM of 3 - 5 rats.

**: Significantly different from HGF alone ($p < 0.01$).

Figure 3. Effect of protamine pretreatment period on the mitogenic effect of HGF (A) and EGF (B) in primary cultured rat hepatocytes.

Parenchymal hepatocytes were cultured for 24 h, and HGF (250 pM) (A) or EGF (500

pM) (B) added at the indicated time periods after the addition of protamine (25 μg/ml). DNA synthesis was assayed as incorporation of ^{125}I-deoxyuridine from 22 to 28 h after HGF addition. The right-most sidebar in each panel shows the results of addition of HGF or EGF at 10 min prior to protamine addition. Each value and vertical bar represents the mean ± SEM of 3 - 5 rats.

*: Significantly different from HGF alone ($p < 0.05$).

Figure 4. Elimination profile of protamine after intravenous administration in rats.

At 24 h after ANIT-intoxication, protamine (1.6 mg/kg) was intravenously given, and the concentration of protamine in plasma determined by Azure A-heparin complex method. Each value and vertical bar represents the mean ± SEM of 3 - 5 rats.

Figure 5. Effect of protamine preperfusion period on hepatic extraction ratio of ^{125}I-HGF (A) and ^{14}C-TCA (B).

After preperfusion with protamine at the indicated concentrations for the indicated time period, ^{125}I-HGF (0.2 μCi/0.2 ml/liver) (A) and ^{14}C-TCA (0.2 μCi/0.2 ml/liver) (B) were injected into the portal vein as a bolus, followed by resection of the liver after 30 sec. Radioactivity in the excised liver was determined and normalized by dose. Data represent means ± SEM of 3 - 5 rats.

* and **: Significantly different from control ($p < 0.05$ and 0.01, respectively).

Figure 6. Binding of protamine (A, B) or HGF (D) to heparin and inhibition by protamine of HGF binding to heparin (C).

In panel A, 20 μL of heparin- sepharose beads were incubated with 500 μg/ml of protamine at 37°C for the indicated period, and binding of protamine was determined. In panel B, the same amount of heparin sepharose beads was incubated with protamine (0.025 - 1.0 mg/ml) at 37°C

for 30 min, and the binding of protamine shown as a Scatchard plot. In panel C, 40 μl of heparin sepharose beads were incubated with HGF (500 μg/ml) alone (●), with protamine simultaneously added (○) or protamine added 10 min prior to HGF addition (■), followed by ELISA determination of bound HGF. In panel D, 40 μl of heparin sepharose beads were incubated with 10 μl of HGF at final concentrations of 100-1000 μg/ml, and the binding of HGF shown as a Scatchard plot. Data represent mean ± SEM of 3 independent experiments.

Figure 7. Effect of protamine on HGF elimination and HGF-induced DNA synthesis in rat hepatocytes.

Parenchymal rat hepatocytes were cultured for 24 h. Protamine at 0 (○) or 25 (●) μg/ml was added 10 min prior to addition of HGF. In panel A, HGF concentration in the medium was determined by ELISA after the addition of 250 pM HGF. Data represent means ± SEM of 3 - 5 rats. In panel B, 50 μl of medium was collected at 0, 1, 3, 6 and 24 h after addition of various concentrations of HGF to determine AUC values. ^{125}I-deoxyuridine was added at 22 h after addition of HGF, and its incorporation for 6 h was assayed as DNA synthesis, shown plotted against AUC of HGF. Data represent means ± SE of 3 - 5 rats.

**: Significantly different from control ($p < 0.01$).