Ligand-induced downregulation of receptor-mediated clearance of hepatocyte growth factor in rats

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Liu, Ke-Xin, Yukio Kato, Ichiro Kino, Toshikazu Nakamura, and Yuichi Sugiyama. Ligand-induced downregulation of receptor-mediated clearance of hepatocyte growth factor in rats. *Am. J. Physiol.* 275 (*Endocrinol. Metab.* 38): E835–E842, 1998.—The change in tissue uptake clearance of ^{125}I-labeled hepatocyte growth factor (HGF) after an intravenous injection of an excess (120 μg/kg) of unlabeled HGF was examined in rats. The heparin-washable component of the hepatic uptake clearance of ^{125}I-HGF was only slightly changed, whereas the heparin-resistant component was significantly reduced 30 min after injection of excess HGF, followed by gradual recovery with a half-life of 3.2 h. Because the former clearance mainly represents ^{125}I-HGF association with heparan sulfate proteoglycan on the cell surface and/or extracellular matrix, whereas the latter includes relatively specific clearance, such as receptor-mediated endocytosis, this result suggests that injection of excess HGF selectively causes downregulation of receptor-mediated HGF clearance in the liver. Downregulation could also be observed for HGF receptor density in isolated liver plasma membrane, assessed by Western blot analysis by means of anti-receptor antibody, 30 min after injection of excess unlabeled HGF, supporting the hypothesis that the overall elimination of HGF from the systemic circulation can be affected by a change in HGF receptor density on the liver cell surface.

receptor-mediated endocytosis; internalization; heparin; c-Met; pharmacokinetics

MANY TYPES OF BIOLOGICALLY ACTIVE POLYPEPTIDES exert their activity through binding to their own receptors on the cell surface. The polypeptide receptor is internalized with its ligand after binding to the ligand. This process is termed receptor-mediated endocytosis (RME) (8, 27). RME is responsible for the systemic clearance of many types of polypeptides (27). Thus the polypeptide receptor acts as a clearance receptor as well as a pharmacological receptor (27). Actually, the silent receptor that acts as an elimination mechanism for polypeptides has been identified for atrial natriuretic factor (16).

In RME, excess ligand in the extracellular space causes the following two phenomena, which affect the efficiency of receptor-mediated clearance of the ligand. One is the saturation of receptor binding on the cell surface, and the other is the reduction in the density of the cell-surface receptors themselves (8). The latter phenomenon is called downregulation, one of the unique characteristics in RME (2, 8, 18). Downregulation of cell-surface receptors has been demonstrated for many types of biologically active polypeptides (2, 4, 17, 18, 21, 23). To give a detailed description of the pharmacokinetics of polypeptides, it is necessary to examine the intracellular kinetics of the receptor as well as the ligand (8).

Changes in growth hormone (GH) receptor density induced by excess GH have been investigated (17, 18). A single injection of GH induces downregulation of the GH receptor in the liver, whereas repeated injections cause very little reduction in receptor density on the cell surface (18). On the other hand, a continuous infusion of an equal amount of GH induces upregulation of GH receptors (17). Thus the receptor density of polypeptides can be affected by the dose of polypeptides used and also by how the polypeptides are exposed to the cells. However, little information has been reported that gives a quantitative analysis of the mechanism for the change in receptor density induced by exposure to ligand.

Hepatocyte growth factor (HGF) was first discovered as a potent mitogen for mature hepatocytes and now is recognized as a mitogen, morphogen, and motogen for a variety of types of epithelial cells (20, 22). Because HGF exhibits marked pharmacological effects in vivo in animal models of liver disease (5, 6, 26) and renal failure (9), it is expected that HGF will be developed as a treatment for certain types of diseases. We previously suggested, using both in vivo and liver perfusion systems, that receptor-mediated endocytosis in the liver contributes to the elimination of HGF from the circulation in rats (12–15). In addition, we found that there is another mechanism with a lower affinity for HGF than RME that is also involved in HGF uptake by the liver (7, 12–15). In an analysis of its nonlinear pharmacokinetics, the low-affinity component for the plasma clearance of HGF was identified, and it was shown that it cannot be completely saturated even when there is a sufficient plasma concentration to occupy the cell-surface receptor (30). We believe that the heparan sulfate proteoglycan (HSPG) on the cell surface and/or extracellular matrix is involved in such nonspecific elimination of HGF, because this substance can bind to HGF (7, 19, 33), and a large amount of cell-surface binding of ^{125}I-labeled HGF can be dissociated by heparin in perfused rat liver (13).

In the present study, we examined the downregulation induced by injection of excess HGF on these two types of clearance mechanisms in the liver. To sepa-

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rately determine the kinetics of the hepatic clearance mediated by such HSPG in vivo, we determined both heparin-washable and heparin-resistant hepatic uptake clearances (12–15). In addition, we directly determined the density of the HGF receptor (protooncogene c-*met* product c-Met) on the liver cell surface by use of Western blot analysis to show that HGF receptor density governs the hepatic elimination of HGF.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 250 g (Nisseizai, Tokyo, Japan) were used. All animals were treated humanely. The studies described in this article 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.

Materials. Porcine intestinal mucosa heparin was from Sigma (St. Louis, MO); rabbit polyclonal antibody recognizing the COOH-terminal 21-amino acid sequence of mouse c-Met, which also cross-reacts with rat c-Met, was from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit antiserum recognizing the NH_2-terminal 16-amino acid sequence of rat Na^+-dependent taurocholate-cotransporting polypeptide (Ntcp) (3) was from BioLink (Tokyo, Japan); human recombinant HGF purified from a culture medium of C-127 cells transfected with plasmid containing human HGF cDNA (22) was radiolabeled with ^{125}I-Na by the chloramine-T method, as described previously (13). The specific activity of ^{125}I-HGF prepared in this way was 70–160 Ci/g.

Induction of receptor downregulation. With rats under light ether anesthesia, excess unlabeled HGF (120 μg/kg body wt) dissolved in saline was administered through the tail vein. Plasma HGF concentrations were determined by an enzyme immunoassay (EIA) kit (Institute of Immunology, Tochigi, Japan).

Measurement of tissue uptake clearance of ^{125}I-HGF. At 10 and 30 min and 1, 2, 3, 6, and 24 h after the induction of downregulation, a tracer amount of ^{125}I-HGF (5 μCi, 0.6 pmol/kg body wt) was administered through the femoral vein under light ether anesthesia. The TCA-precipitable radioactivity in plasma was determined as described previously (13, 14). At 10 min after intravenous administration of ^{125}I-HGF, a piece (100 mg tissue) of the liver was removed by biopsy (14). Eleven minutes after ^{125}I-HGF administration, heparin (100 mg/kg body wt) dissolved in saline (1 ml/kg body wt) was injected through the opposite side of the femoral vein, and plasma samples were collected (14). Four minutes after the injection of heparin, rats were killed, and the liver, adrenal, spleen, kidney, lung, duodenum, and muscle were excised (14). Both the liver biopsy specimen and an aliquot of each tissue were weighed and counted (14).

The plasma concentration-time profiles of TCA-precipitable ^{125}I-HGF before the injection of heparin were fitted to the following biexponential equation by use of a nonlinear iterative least squares method (14)

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

where C_p is the plasma concentration of TCA-precipitable radioactivity, A and B are the corresponding *time 0* intercepts, α and β are the apparent rate constants, and t is time.. The plasma clearance (CL_{plasma}) was calculated as follows (13)

$$CL_{plasma} = \text{dose}/AUC = \text{dose}/(A/\alpha + B/\beta) \quad (2)$$

The heparin-resistant component of tissue uptake clearance ($CL_{uptake,HR}$) was obtained by

$$CL_{uptake,HR} = X_t/AUC_{(0\text{-}10)} \quad (3)$$

where X_t is the amount of heparin-resistant radioactivity in the tissue determined after the heparin injection, and $AUC_{(0\text{–}10)}$ is the area under the plasma concentration-time curve from *time 0* to 10 min, calculated from

$$AUC_{(0\text{-}10)} = \int_0^{10} C_p \, dt = A/[1 - \exp(-10\alpha)] + B/[1 - \exp(-10\beta)] \quad (4)$$

For the liver, the heparin-washable component of the hepatic uptake clearance ($CL_{uptake,HW}$), which probably represents HGF binding to HSPG on the cell surface and/or extracellular matrix (14), was calculated from

$$CL_{uptake,HW} = CL_{uptake,HW+HR} - CL_{uptake,HR} \quad (5)$$

where $CL_{uptake,HW+HR}$ was determined from *Eq. 3*, where X_t is the radioactivity in the liver biopsy sample removed before heparin injection.

Calculation of the recovery half-life of tissue uptake clearance. For the data in the recovery phase, from 30 min to 24 h after induction of downregulation, the values of $\ln[CL_{uptake,HR}(\text{control}) - CL_{uptake,HR}(t)]$ were plotted vs. time (Sigma-minus plot), where $CL_{uptake,HR}$(control) represents the values for $CL_{uptake,HR}$ determined in the control rats, and $CL_{uptake,HR}(t)$ represents the values for $CL_{uptake,HR}$ determined at *time t* after intravenous administration of an excess of unlabeled HGF. The recovery half-life [$t_{1/2\,(recovery)}$] was calculated from the slope of the linear regression line of the Sigma-minus plot as follows (32)

$$t_{1/2\,(recovery)} = \ln 2/\text{slope} \quad (6)$$

Western blot analysis of the HGF receptor in isolated plasma membrane. Rat liver plasma membrane was separated as reported (25) and stored as a solution, 1 mg protein/ml, at −100°C. Each membrane preparation originates from one rat liver, and three independent series of experiments were performed, each experiment series using one untreated rat and a rat each at 30 min and 24 h after induction of downregulation. Marker enzyme activities such as Na^+-K^+-ATPase, acid phosphatase, and glucose-6-phosphatase were determined by the method of Yachi et al. (31), an assay kit for acid phosphatase (Wako Pure Chemical Industries, Osaka, Japan), and Yachi et al. (31), respectively, to check the purity of the prepared plasma membranes. An aliquot, 16–30 μl, of the stored membrane was centrifuged at 20,000 *g* (Optima TLX, Beckman Instruments, Fullerton, CA) at 4°C for 10 min. The supernatant was discarded, and the precipitate was solubilized with 30 μl of Laemmli buffer (11). The solubilized sample (10 μl) was subjected to SDS-PAGE under reducing conditions with a separating gel containing 5% acrylamide and then was transferred onto Immobilon-P (Millipore, Tokyo), which was subsequently treated with anti-c-Met antibody or antiserum recognizing Ntcp. The Immobilon-P was then incubated with an ^{125}I-anti-rabbit immunoglobulin $F(ab')_2$ fragment (Amersham, Arlington Heights, IL), and blots were quantified using a Bio-Image Analyzer (BAS-2000, Fuji Photo Film, Tokyo, Japan). The background values were subtracted from the amounts measured on the blots. All values were expressed as a percentage of the immunoreactive

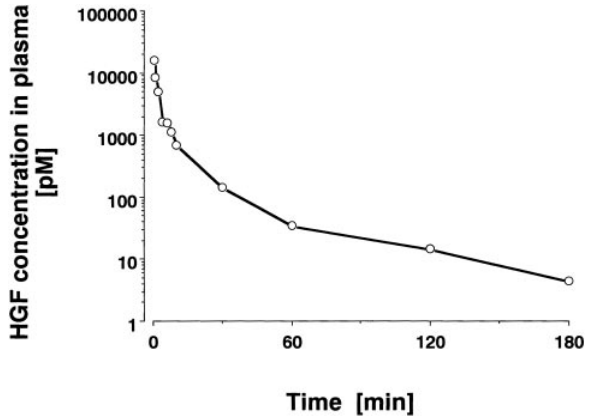

Fig. 1. Time profiles of plasma hepatocyte growth factor (HGF) concentrations after iv administration of excess unlabeled HGF (120 μg/kg body wt). Plasma concentrations were determined using an enzyme immunoassay (EIA) kit. Each point represents the mean of 3 different rats; SE values were smaller than symbols for all data points.

amount that came from 10 μg of membranous protein prepared from untreated (control) rats.

RESULTS

Plasma concentration-time profile of HGF after an intravenous administration of excess unlabeled HGF as a bolus. After intravenous administration of unlabeled HGF (120 μg/kg), its plasma concentration-time profile was determined using EIA (Fig. 1). Plasma HGF rapidly disappeared after being given intravenously and exhibited concentrations of 145 ± 37, 35.1 ± 5.3, 14.5 ± 1.9, and 4.39 ± 1.01 pM at 30 min and 1, 2, and 3 h after administration (Fig. 1).

Change in tissue uptake clearance and plasma clearance of ^{125}I-HGF after intravenous administration of excess unlabeled HGF. After an intravenous administration of excess unlabeled HGF (120 μg/kg), a tracer amount of ^{125}I-HGF was subsequently given to determine its tissue uptake clearance and plasma clearance (Figs. 2-4). At 10 min after the injection of excess HGF, only a slight change in the $CL_{uptake,HR}$ of ^{125}I-HGF could be observed for the liver, adrenal, spleen, kidney, lung and duodenum compared with control levels (the values in untreated rats) (Fig. 2), whereas at 30 min after injection of excess HGF, the $CL_{uptake,HR}$ for these organs was significantly reduced (Fig. 2). The $CL_{uptake,HR}$ for the liver at 30 min after injection of excess unlabeled HGF was 44% that of the control level (Fig. 2). The CL_{plasma} was determined from TCA-precipitable radioactivity in plasma after injection of a tracer amount of ^{125}I-HGF and was also reduced 30 min after injection of excess HGF (Fig. 2). When a tracer amount of ^{125}I-HGF was coadministered with excess HGF (120 μg/kg), both CL_{plasma} and $CL_{uptake,HR}$ in the liver exhibited very little reduction and were not significantly different from the control values (Fig. 2). The $CL_{uptake,HW}$ in the liver did not show any significant change either after injection of excess HGF or after coinjection of excess HGF and the tracer ^{125}I-HGF (Fig. 2).

In the liver, the $CL_{uptake,HR}$ gradually recovered, starting 30 min after injection of excess unlabeled HGF (Fig. 3). When both the $CL_{uptake,HW}$ and $CL_{uptake,HR}$ were calculated per kilogram body weight values, the sum of these clearances was 85, 89, and 86% of the CL_{plasma} at baseline and 30 min and 24 h after injection of excess HGF (Fig. 3).

The $CL_{uptake,HR}$ for extrahepatic organs such as the adrenal, spleen, kidney, lung, and duodenum exhibited minimum values around 30 min after injection of excess unlabeled HGF, followed by a gradual recovery (Fig. 4). On the other hand, the $CL_{uptake,HR}$ for muscle did not show any clear reduction or subsequent recovery (Fig. 4). The $CL_{uptake,HR}$ $t_{1/2(recovery)}$ was calculated from Sigma-minus plots shown in Fig. 5. The $t_{1/2(recovery)}$ in the liver was 3.15 h, comparable with the $t_{1/2(recovery)}$

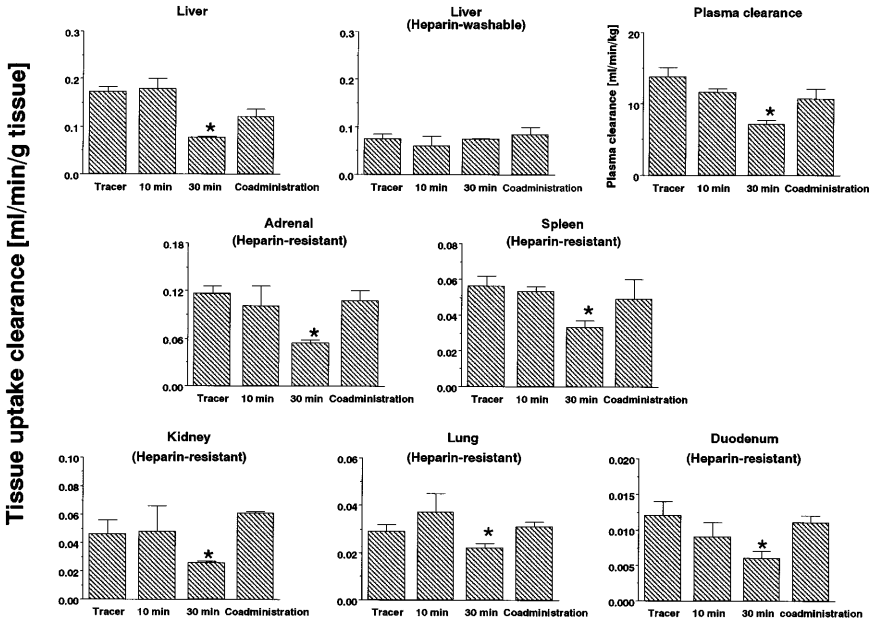

Fig. 2. Time-dependent reduction in tissue uptake clearance of ^{125}I-labeled HGF after iv administration of excess unlabeled HGF. At 10 (2nd bar) or 30 min (3rd bar) after iv administration of excess unlabeled HGF (120 μg/kg body wt), a tracer amount of ^{125}I-HGF was also administered, and heparin-resistant tissue uptake clearance ($CL_{uptake,HR}$) for the indicated organs, heparin-washable clearance ($CL_{uptake,HW}$) for the liver, and plasma clearance (CL_{plasma}) of ^{125}I-HGF were determined. First bar, those values obtained without previous injection of unlabeled HGF (control values); 4th bar, those values obtained after coadministration of excess HGF with a tracer amount of ^{125}I-HGF, respectively. Values are means ± SE of 3 rats. *Significantly different from control values ($P < 0.05$).

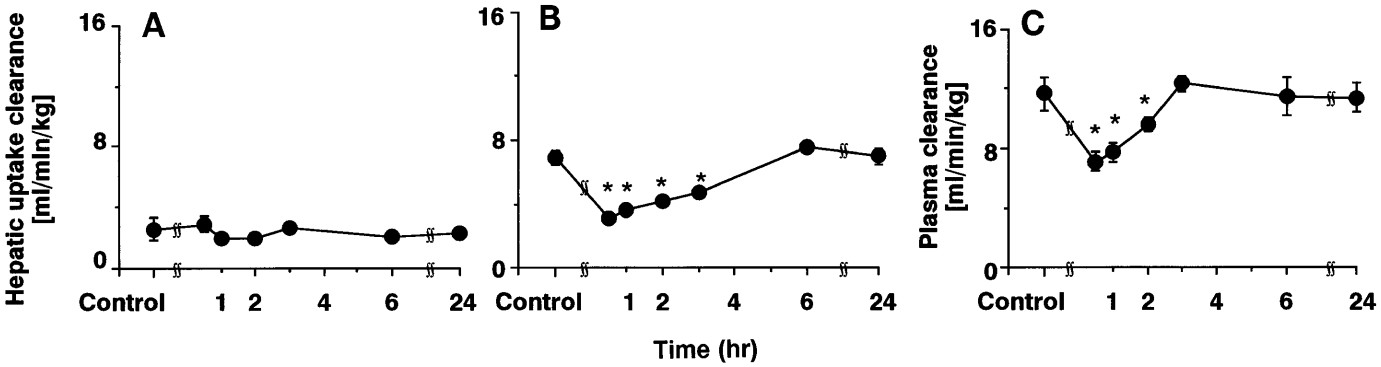

Fig. 3. Change in hepatic uptake clearance and plasma clearance of ^{125}I-HGF after iv administration of excess unlabeled HGF. At specified times after iv administration of excess unlabeled HGF (120 μg/kg body wt), a tracer amount of ^{125}I-HGF was also administered. Both $CL_{uptake,HW}$ (*A*) and $CL_{uptake,HR}$ (*B*) in liver, and CL_{plasma} of ^{125}I-HGF (*C*) were determined. Each point and vertical bar represent mean ± SE of 3 different rats. * Significantly different from control values ($P < 0.05$).

values for the adrenal, spleen, lung and duodenum (3.28, 2.97, 1.85, and 2.65 h, respectively; Fig. 5).

Change in HGF receptor density in isolated plasma membrane from liver after injection of excess unlabeled HGF. At 30 min and 24 h after injection of excess unlabeled HGF (120 μg/kg), plasma membranes were isolated from the livers and solubilized to determine their HGF receptor content by Western blot analysis (Fig. 6). The immunoreactive band was observed at 140 kDa (Fig. 6*A*). This band was reduced 30 min after injection of unlabeled HGF compared with that in untreated rats [62.1 ± 4.5% (SE) that in untreated rats of three independent series of experiments; Fig. 6*A*], followed by its recovery to the control level at 24 h after the HGF injection (110 ± 19% that in untreated rats). As a control experiment, Ntcp content in the liver plasma membranes was also determined (Fig. 6*A*). The 49-kDa immunoreactive band was observed, and the change in the intensity of this band was minimal after the injection of unlabeled HGF (98.5 ± 8.5 and 83.8 ± 12.3% of that in normal liver at 30 min and 24 h after injection of unlabeled HGF; Fig. 6*A*). To confirm the validity of this quantification of receptor density by Western blotting, the linearity of the intensity of the 140-kDa band was investigated by varying the amount of membranous protein prepared from untreated liver and subjected to electrophoresis (Fig. 6*B*). A significant ($P < 0.05$) correlation could be observed between the intensity of the 140-kDa band and the membranous protein subjected to electrophoresis (Fig. 6*B*).

Purity of isolated plasma membranes. To check the purity of the plasma membranes, we examined the relative enrichment (ratio of specific activity in membranes to the specific activity in homogenate) of the marker enzymes for plasma membrane (Na^+-K^+-ATPase), lysosomes (acid phosphatase), and microsomes (glucose-6-phosphatase). The enrichment for Na^+-K^+-ATPase was 71.6 ± 17.0, 89.9 ± 3.1, and 72.6 ± 14.5 (means ± SE of three independent series of experiments) in the control condition and at 30 min and 24 h after HGF injection, respectively. The enrichment for acid phosphatase was 1.32 ± 0.07, 1.23 ± 0.17, and 1.34 ± 0.19, whereas that for glucose-6-phosphatase was 1.35 ± 0.16, 1.48 ± 0.14, and 1.11 ± 0.10 in the control condition and at 30 min and 24 h after HGF injection, respectively. Thus enrichment of the plasma membrane fraction was much higher than that of the other intracellular organella.

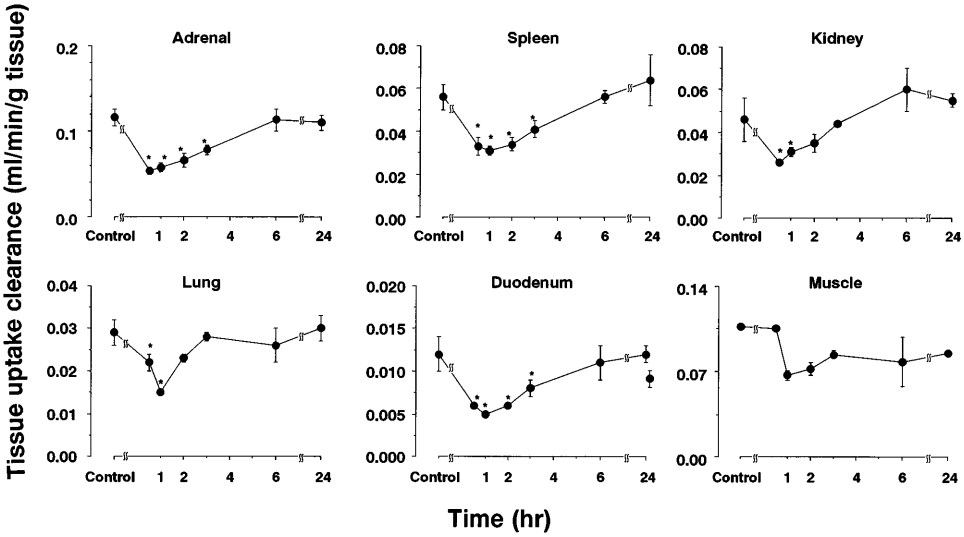

Fig. 4. Change in tissue uptake clearance of ^{125}I-HGF after iv administration of excess unlabeled HGF (120 μg/kg body wt). At specified times, a tracer amount of ^{125}I-HGF was also administered, and $CL_{uptake,HR}$ of ^{125}I-HGF in extrahepatic organs was determined. Each point and vertical bar represent mean ± SE of 3 different rats. * Significantly different from control values ($P < 0.05$).

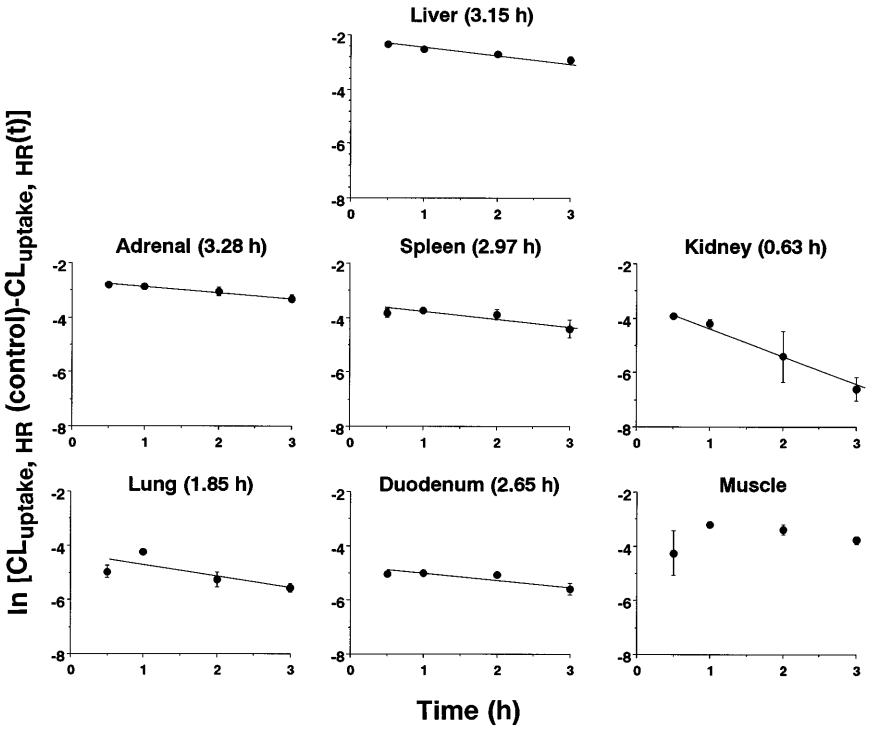

Fig. 5. Sigma-minus plots for determination of the recovery half-life [$t_{1/2(recovery)}$] values of $CL_{uptake,HR}$. Data shown in Figs. 3 and 4 were used to calculate the $t_{1/2(recovery)}$ values of $CL_{uptake,HR}$ of ^{125}I-HGF by use of *Eq. 6*. Each point and vertical bar represent mean ± SE of 3 different rats. Half-lives obtained are shown in parentheses.

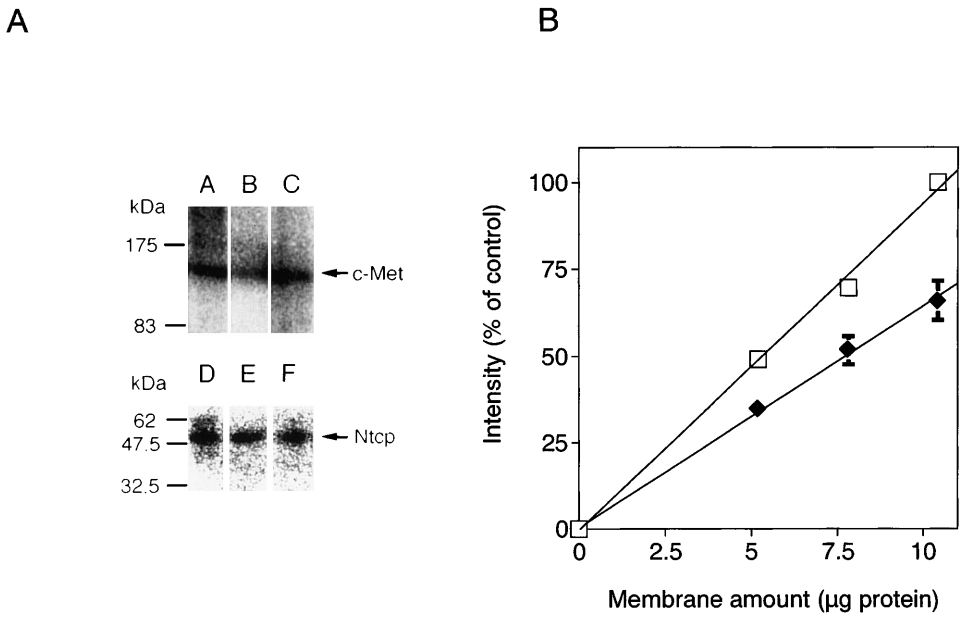

Fig. 6. Western blot analysis of HGF receptor density in isolated plasma membranes from liver after ligand-induced downregulation. *A*: in an untreated rat (*lanes A* and *D*) and rats 30 min (*lanes B* and *E*) and 24 h (*lanes C* and *F*) after iv administration of excess unlabeled HGF (120 μg/kg body wt), plasma membrane was isolated from liver and solubilized. Solubilized samples (10 μg protein) were subjected to SDS-PAGE and then transferred onto Immobilon-P, which was subsequently treated with anti-c-Met antibody (*lanes A*, *B*, and *C*) or anti-Na^+-dependent taurocholate-cotransporting polypeptide (Ntcp) antiserum (*lanes D*, *E*, and *F*), and then with ^{125}I-labeled anti-rabbit immunoglobulin $F(ab')_2$ fragment. Values shown are typical examples of results, and reproducible results could also be obtained in another two series of experiments. *B*: plasma membrane was isolated from an untreated rat (□) and a rat 30 min (◆) after iv administration of excess unlabeled HGF (120 μg/kg body wt). Solubilized membranes (0, 5.2, 7.8, and 10 μg protein) were then subjected to SDS-PAGE and Western blot analysis using anti-HGF receptor antibody. Intensity of the 140-kDa band was quantified using a Bio-Image Analyzer and plotted against the amount of membranous protein applied to SDS-PAGE. Values are means ± SE of 3 different rats shown as a % of the immunoreactive amount from 10 μg of membranous protein prepared from an untreated rat. Straight line, linear regression line ($P < 0.05$).

DISCUSSION

We have previously suggested that the elimination mechanism of HGF from circulating plasma consists of two components in the liver; one is RME, and the other is a nonspecific uptake mechanism probably mediated by a cell-surface HSPG (12–15). If RME is involved in hepatic clearance, decrease in this clearance should be observed, resulting from downregulation of HGF receptors on the liver cell surface. In the present study, we confirmed that excess unlabeled HGF given intravenously to rats induces receptor downregulation, which affects both hepatic uptake clearance and plasma clearance of a tracer amount of ^{125}I-HGF.

HGF receptors on the liver cell surface were downregulated by injection of excess unlabeled HGF. Such receptor downregulation was confirmed by Western blot analysis (Fig. 6). The $CL_{uptake,HR}$ for the liver, a major clearance organ for HGF (13), was also significantly reduced by an injection of excess HGF, followed by a gradual recovery, whereas the $CL_{uptake,HW}$ did not show any clear change (Fig. 3). We have previously identified two types of cell surface-associated ^{125}I-HGF in perfused rat liver after perfusion of tracer concentrations: heparin-washable, and heparin-resistant acid-washable ^{125}I-HGF (13). The former has a lower affinity for HGF than the latter and may represent ^{125}I-HGF bound to HSPG on the cell surface and/or extracellular matrix (13). The latter mainly represents receptor binding of ^{125}I-HGF, because this binding could be reduced by coperfusion with excess unlabeled HGF (13). The radioactivity was still present in liver after both heparin and acid washing and could be partially reduced either by coperfusion with excess HGF or an inhibitor of RME, phenylarsine oxide; however, approximately one-half of this radioactivity could not be inhibited (13). Therefore, HGF is taken up by the liver through both RME and another nonspecific uptake mechanism, which has a lower affinity for HGF and cannot be inhibited by either excess HGF or inhibitor (13). On the basis of these findings, the $CL_{uptake,HW}$ should reflect ^{125}I-HGF binding to HSPG, whereas $CL_{uptake,HR}$ should reflect its receptor binding and subsequent internalization in the liver. Therefore, the $CL_{uptake,HR}$ includes receptor-mediated clearance of ^{125}I-HGF. This is suggested by the following findings. *1*) Only the $CL_{uptake,HR}$ was reduced, whereas the $CL_{uptake,HW}$ was not (Fig. 3). *2*) HGF receptor density in the liver plasma membrane, assessed by Western blot analysis, was also reduced (Fig. 6), and the degree of such reduction was almost comparable for both $CL_{uptake,HR}$ and receptor density (44 and 62% of the control level, respectively) 30 min after injection of excess unlabeled HGF. These results suggest that receptor-mediated clearance in the liver is decreased because of the receptor downregulation induced by injection of excess unlabeled HGF.

Tajima et al. (30) showed that plasma membranes from liver, adrenal, spleen, kidney, and lung exhibited specific ^{125}I-HGF binding in rats. Prat et al. (24) reported that immunoreactive protein against the HGF receptor antibody was detected in liver and jejunum of humans, although such protein was undetectable in spleen, kidney, and lung. These reports imply that the HGF receptor is expressed in these organs. For these organs in the present study, the $CL_{uptake,HR}$ showed reduction and subsequent recovery (Fig. 4). On the other hand, no specific binding of ^{125}I-HGF could be detected for plasma membrane of muscle (30), and no immunoreactive protein was detected for skeletal and smooth muscle (24). Therefore, the HGF receptor density in muscle is undetectable; hence, no clear reduction was observed for the $CL_{uptake,HR}$ in muscle (Figs. 4 and 5).

After injection of excess unlabeled HGF, both $CL_{uptake,HW}$ and $CL_{uptake,HR}$ in the liver were calculated in terms of kilogram body weight (Fig. 3). The sum of these values was close to the CL_{plasma} any time after injection of unlabeled HGF (Fig. 3), suggesting that the liver is a major clearance organ for HGF after the induction of receptor downregulation. As indicated in Fig. 3, the reduction and subsequent recovery of CL_{plasma} can be attributed to the reduction and recovery of $CL_{uptake,HR}$ in the liver. Such a reduction and subsequent recovery of the $CL_{uptake,HR}$ result from the downregulation and subsequent recovery of HGF receptors on the liver cell surface, as we have discussed. Therefore, these results demonstrate that receptor downregulation in the liver has a critical effect on the overall elimination of HGF from the circulating plasma.

In the present study, the $CL_{uptake,HR}$ in the liver was not significantly reduced at 10 min, whereas a significant reduction occurred 30 min after injection of excess unlabeled HGF (Fig. 2). We previously reported that the half-life of HGF internalization is 7–11 min in perfused rat liver (14, 15, 29). When excess unlabeled HGF was injected intravenously as a bolus, most of the cell-surface receptors can be occupied by HGF and subsequently become internalized, resulting in a transient reduction in HGF receptor density on the cell surface (Fig. 6). If the binding and subsequent internalization occur in such a gradual manner, a gradual reduction in $CL_{uptake,HR}$ for the liver (Fig. 2) can be explained.

The $CL_{uptake,HR}$ in the liver, adrenal, spleen, kidney, lung, and duodenum showed minimum values around 30 min after injection of excess unlabeled HGF (Figs. 3 and 4). At this time the plasma concentration of unlabeled HGF was still 145 pM (Fig. 1). Therefore, the reduction in $CL_{uptake,HR}$ could be explained if we assume that any unlabeled HGF remaining in the circulating plasma competitively inhibits the tissue uptake of ^{125}I-HGF. However, this hypothesis can be refuted, because the reduction in $CL_{uptake,HR}$ was minimal when a tracer amount of ^{125}I-HGF was simultaneously injected with excess unlabeled HGF (Fig. 2). Although a slight amount of saturation was observed for the $CL_{uptake,HR}$ in the liver and CL_{plasma}, the reduction after coadministration of excess unlabeled HGF was very small and not significant, compared with the reduction 30 min after injection of excess unlabeled HGF (Fig. 2). If we assume that the unlabeled HGF remaining in the

circulating plasma competitively inhibits tissue uptake of a tracer amount of ^{125}I-HGF, the reduction after coadministration of excess unlabeled HGF should be more marked than that 30 min after injection of excess HGF, because the remaining HGF level in circulating plasma was higher after coadministration than at 30 min. In addition, the $CL_{uptake,HR}$ for the liver and other extrahepatic organs 10 min after injection of excess unlabeled HGF was still higher than for those organs at 30 min (Fig. 2). For the same reason, as we have mentioned, this result also suggests that the decrease in the $CL_{uptake,HR}$ found at 30 min cannot be attributed only to competitive inhibition of ^{125}I-HGF uptake by the unlabeled HGF remaining in the circulation.

We have previously investigated the change in tissue uptake clearance of a tracer amount of ^{125}I-labeled epidermal growth factor (EGF) after intravenous administration of excess unlabeled EGF (32). We here would like to compare the downregulation of EGF and HGF receptors. According to our analysis, the $t_{½(recovery)}$ of the hepatic uptake clearance of ^{125}I-EGF is 22 min, much higher than that for other extrahepatic organs such as spleen, kidney, duodenum, and stomach (2–5 h) (32). The major clearance organ for EGF is also the liver (10). Hepatocytes highly express EGF receptors (100,000–150,000 sites/cell) (28), and RME via the receptors is responsible for the hepatic elimination of EGF (10). Also, in extrahepatic organs such as kidney, spleen, stomach, duodenum, and jejunum, EGF is taken up by a saturable uptake mechanism representing RME (10). Therefore, such a rapid recovery of hepatic EGF uptake, compared with extrahepatic organs, suggests the importance of RME in the liver as a homeostatic regulator that maintains the level of circulating EGF. The recruitment of cell-surface receptors is rapid in the liver so that RME in the liver can quickly recover to maintain the EGF concentration in plasma at an appropriate level. The liver is also a major clearance organ for HGF (13). Nevertheless, the $t_{1/2(recovery)}$ for $CL_{uptake,HR}$ in the liver is 3.2 h, comparable with that for extrahepatic organs (1–3 h) (Fig. 5). As we have mentioned, the hepatic clearance mechanism for HGF involves not only RME but also another nonspecific uptake mechanism, probably mediated by cell-surface HSPG (12–15). The receptor-mediated clearance in the liver is decreased not only by injection of excess unlabeled HGF (Figs. 2 and 3) but also by the administration of a hepatotoxin such as CCl_4 (15) and by partial hepatectomy (14). The reduction in receptor-mediated clearance in such cases also results from receptor downregulation (3, 14, 15). However, the nonspecific uptake mechanism is not downregulated and is still functional both after injection of excess HGF (Fig. 3) and after hepatic damage (14, 15). Because there is an uptake mechanism other than RME, the HGF level in circulating plasma may be under control even when the receptor-mediated clearance has not recovered very rapidly from its downregulation.

The $CL_{uptake,HR}$ at 30 min after HGF injection was 44% of control (Fig. 3*B*), whereas the receptor density in plasma membrane at that time was 62% of control (Fig. 6). One of the possible explanations for such a slight difference is that, at 30 min after HGF injection, a fraction of cell-surface receptors was still bound to unlabeled HGF and could not bind to tracer ^{125}I-HGF. This could result in a reduction in $CL_{uptake,HR}$; however, such receptors on the cell surface should be detected by Western blot analysis. Another possible explanation is contamination of the plasma membrane preparations by intracellular vesicles. Although the enrichment of Na^+-K^+-ATPase is much higher than that of other marker enzymes, a small degree of contamination by intracellular vesicles may affect the receptor density in plasma membranes assessed by Western blot analysis. After the injection of excess HGF, a fraction of the cell-surface receptors is internalized, possibly resulting in the increase in receptor density in intracellular vesicles. Therefore, the impact of such contamination on the assessment of receptor density in plasma membrane is more marked at 30 min after HGF injection than in control rats. It could also be that some of the receptors on the cell surface at 30 min after HGF injection were functionally inactive and could not bind to HGF. However, such "inactive" receptors can still be detected by Western blot analysis and have a molecular mass similar to the active receptor (140 kDa). To examine the validity of a such possibility, equilibrium binding studies and Scatchard analysis with ^{125}I-HGF are necessary. In fact, we have tried to perform such analysis but could not get a clear result, probably because of the high degree of nonspecific binding of ^{125}I-HGF to the membranes. Further studies are needed to clarify whether all of the HGF receptors remaining in the plasma membrane after the induction of downregulation are functionally active.

In conclusion, *1*) injection of excess unlabeled HGF induces receptor downregulation in the liver, resulting in a transient reduction in receptor-mediated clearance, concomitantly with a transient reduction in plasma HGF clearance, and *2*) the recovery half-life of receptor-mediated uptake is 1–3 h, independent of the organs. The present study demonstrates that HGF receptors are directly involved in the systemic clearance of HGF.

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