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KINETIC ANALYSIS OF THE ELIMINATION PROCESS OF HUMAN EPIDERMAL GROWTH FACTOR (hEGF) IN RATS*

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Abstract—Pharmacokinetic study of human epidermal growth factor (hEGF) in rats was performed *in vivo*. The hepatic extraction ratio (E_H) of [^{125}I]hEGF, determined from the difference between the artery and the hepatic vein plasma concentrations at steady state, was 0.19. The hepatic clearance (CL_H: 7.56 ml/min/kg body wt), calculated by multiplying E_H by the hepatic plasma flow rate ($Q_{P,H}$), was approximately 70% of the total body clearance (CL_{tot}: 10.8 ml/min/kg body wt), which was determined from the steady-state arterial plasma concentration and the infusion rate. These results indicated that the liver is the main organ responsible for the removal of [^{125}I]hEGF from the systemic circulation in rats. The renal extraction ratio (E_R) of [^{125}I]hEGF was half of that of [^{14}C]inulin; this may have resulted from the plasma protein binding of [^{125}I]hEGF, which was approximately 50% as determined by the charcoal adsorption method and the equilibrium gel-filtration method. The renal clearance (CL_R: 2.65 ml/min/kg body wt), calculated by multiplying E_R by the renal plasma flow rate (Q_{PR}), was approximately 17% of the CL_{tot} (15.6 ml/min/kg body wt), indicating a minor contribution of CL_R to CL_{tot} compared with that of CL_H to CL_{tot}. The CL_R of [^{125}I]hEGF calculated from the urinary excretion data was one-tenth of that calculated from the plasma concentration difference between the femoral artery and the renal vein at steady state. These results suggest that the bulk of [^{125}I]hEGF cleared from the plasma by the kidney may have been metabolized further in the renal tubules before appearing in the urine.

Macromolecules are gaining increasing importance as therapeutic agents. But our understanding of their pharmacokinetic behaviors is limited, even though it is important to predict both the concentration–time profiles of macromolecules at receptor sites and the physiological action of macromolecules. As a model macromolecule, we selected epidermal growth factor (EGF), which is known to bind to its specific receptor on the cell surface membrane [1] and to endocytose into the intracellular compartment and then degrade by lysosomal enzyme [2].

There are many reports suggesting that the binding of EGF to its specific receptors induces the various biological responses *in vitro* [3–5]. However, little information is available about the regulation of EGF plasma concentration in the intact animal, though it is important for the control of biological responses in the target organs.

We previously reported that the liver and kidney are the main organs responsible for the early distribution of hEGF after intravenous bolus administration in rats, and that the binding of hEGF to these organs is via a saturable process [6]. We then undertook the *in vivo* study to investigate further the contribution of liver and kidney to the elimination and/or metabolic process at steady state.

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MATERIALS AND METHODS

Chemicals

Biosynthetic human epidermal growth factor (hEGF), obtained from *Escherichia coli* via the synthesized coding sequence described previously [7], was used in all experiments. Sodium iodide-125 (100 mCi/ml) was purchased from the Radiochemical Center (Amersham Corp., Arlington Heights, IL, U.S.A.). The hEGF was radiolabeled with [^{125}I]Na by the chloramine-T method [8]. Unreacted [^{125}I]Na was removed by a Sephadex G-25 column, and the [^{125}I]hEGF was eluted in the void volume. The [^{125}I]hEGF had a specific activity of 0.5 to 1.0 mCi/nmol and was >95% precipitable in 15% trichloroacetic acid (TCA). Furthermore, >98% of [^{125}I]hEGF binding to a specific antiserum was displaced by an excess amount of unlabeled hEGF (6 nM) and showed specific binding to its receptor in rat liver homogenates [9], isolated perfused rat liver [10] and rat liver *in vivo* [6]. The preparation of antiserum was reported previously [11]. [^{14}C]Inulin (2.6 μCi/mg) was purchased from ICN Radiochemicals (Division of ICN Biomedicals, Irvine, CA, U.S.A.), and the purity, examined by gel-filtration (Sephadex G-25), was more than 98.5%. Dextran T-70, Sephadex G-25 and Sephadex G-75 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Bovine serum albumin (BSA, Fraction V) and activated charcoal were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were commercially available and of analytical grade.

Administration of [^{125}I]hEGF

Adult male Wistar rats (Nihon Seibutsu Zairyo, Tokyo, Japan) weighing 230–300 g were used throughout the experiments. Food and water were available *ad lib.* Under light ether anesthesia, the jugular vein, the femoral artery, and the common bile duct were cannulated with polyethylene tubing (PE-50) filled with isotonic saline. For the hepatic portal vein cannulation, a venous injection needle (25-gauge, JIS type V3, French scale 7), 11.5 mm length, was connected to polyethylene tubing (PE-50), was inserted into the hepatic portal vein, and was fixed with surgical glue (Aron Alpha, Sankyo Co. Ltd., Japan).

After recovery from ether anesthesia, a tracer dose of [^{125}I]hEGF (8–10 μCi/kg body wt) was dissolved in 300 μl of rat plasma, and this solution was administered through the jugular vein cannula (i.v.) or administered through the hepatic portal vein cannula (h.p.v). Blood samples were withdrawn through the femoral artery cannula at designated times (1, 2, 3, 5, 7, 10, 20, 30 and 60 min) after i.v. or h.p.v. administration, and bile samples were collected at 20-min intervals after i.v. or h.p.v. administration.

Infusion experiments

Under light ether anesthesia, the abdomen was opened through a midline incision, and the renal vein and ureters were exposed. The ureters were cannulated with polyethylene tubing (PE-10) filled with isotonic saline. The renal vein was cannulated with the same kind of cannula that was used for the hepatic portal vein. The jugular vein, the femoral artery, and the femoral vein were cannulated with PE-50 polyethylene tubing filled with isotonic saline. Tracer doses of [^{125}I]hEGF (8–10 μCi/kg body wt) and [^{14}C]inulin (12 μCi/kg body wt), dissolved in 300 μl of pooled rat plasma, were administered into the jugular vein, and then the intravenous infusion of a solution containing both [^{125}I]hEGF and [^{14}C]inulin was started immediately through the femoral vein cannula. The infusion rates were 0.2 μCi/min/kg body wt for both [^{125}I]hEGF and [^{14}C]inulin. For the renal venous blood sampling, a 1-ml syringe was connected to the renal vein cannula, and 350 μl of renal venous blood was drained by pulling the syringe very gently (350 μl blood/30 sec). The hepatic vein cannulation was carried out according to the method of Yokota *et al.* [12]. In the hepatic vein sampling experiment, the bile duct and the hepatic vein were cannulated. Both the administration of [^{125}I]hEGF and [^{14}C]inulin into the jugular vein and the infusion into the femoral vein were done in the same way as in the renal vein sampling experiment. The hepatic venous blood was sampled in the same way as in the renal venous blood sampling.

Plasma binding experiments

The plasma protein binding of hEGF was determined by two methods, namely, the activated charcoal adsorption method and the equilibrium gel-filtration method, which were used successfully in the determination of the plasma binding of β-endorphin [13].

Charcoal adsorption method. Tracer [^{125}I]hEGF and unlabeled hEGF (10, 100 and 1000 nM) were incubated with 0.3 ml of pooled rat plasma or standard buffer (0.13 M phosphate buffer containing 0.1% BSA) at 37° for 60 min. The presence of 0.1% BSA in the standard buffer avoids the adsorption of the peptide to the test tube wall. According to the method of Heyns *et al.* [14], adsorption of the peptide from the solutions by a solid adsorbent was performed by addition of 1 ml of standard buffer containing 6 mg/ml activated charcoal and 0.6 mg/ml dextran T-70. After standing at 4° for 30 min, the adsorbent was centrifuged, and the radioactivities of [^{125}I]hEGF were determined in both the supernatant fraction and the precipitate in an Aloka ARC-300 gamma counter (Aloka Co., Tokyo, Japan) with a counting efficiency of approximately 80%. The adsorption constant *(K)* of [^{125}I]hEGF to charcoal was calculated as K = (cpm in precipitate)/(cpm in supernatant), obtained in the standard buffer solution (without plasma). Binding of the peptide to plasma was calculated as % bound = $(1 - (1/K)\cdot$ (cpm in precipitate)/(cpm in supernatant)) $\times$ 100, obtained in plasma solutions.

Equilibrium gel-filtration method. Equilibrium gel filtration was performed by using the method of Hummel and Dreyer [15] with slight modification. A Sephadex G-75 column (0.9 $\times$ 28 cm) was equilibrated and eluted with the standard buffer containing 16,250 cpm/ml [^{125}I]hEGF (designated as the elution buffer) at 4°. The presence of 0.1% BSA in the elution buffer avoids the adsorption of the peptide to the gel matrix. A 0.5-ml pooled rat plasma to which 0.1% (w/v) BSA had been added was incubated with [^{125}I]hEGF (16,250 cpm/ml) at 37° for 60 min and eluted on a Sephadex G-75 column with the elution buffer. The flow rate was approximately 10 ml/hr, and the fraction volume was 0.8 ml. Absorbance at 280 nm and radioactivity in each fraction were determined. The amount of bound [^{125}I]hEGF to plasma proteins was measured as the area of the peak. Binding of [^{125}I]hEGF to plasma proteins in the equilibrated column was calculated as % bound = (area of the peak)/((area of the peak) + (area under the baseline)) $\times$ 100.

Treatment of samples

Radioactivities of [^{125}I]hEGF in the plasma, urine and bile samples were determined by the method of TCA precipitation right after the sampling. In the case of coadministration of [^{125}I]hEGF and [^{14}C]inulin, the plasma, bile, and urine samples were divided into two fractions for the determination of [^{125}I]hEGF and [^{14}C]inulin respectively. Radioactivity of [^{14}C]inulin was determined in a Tri-Carb liquid scintillation spectrophotometer (model 3255, Packard Instruments, Downers Grove, IL). Determinations were corrected for [^{125}I] radioactivity.

TCA precipitation method

Plasma, bile, and urine samples (50–100 μl) were added to 1 ml of ice-cold 15% (w/v) trichloroacetic acid, containing 0.1% (w/v) bovine serum albumin, and mixed well. After standing at 4° for 30 min, the mixture was centrifuged in a table-top microfuge (Beckman Instruments, Fullerton, CA) for 2 min,

and the supernatant fraction was transferred to another test tube by aspiration. [^{125}I] Radioactivity in both the supernatant fraction and the precipitate were determined using a gamma-counter. The percent of [^{125}I] radioactivity in the precipitate (designated as TCA precipitable %) was calculated as 100 × (cpm in precipitate)/((cpm in precipitate) + (cpm in supernatant)).

Gel filtration of [^{125}I]hEGF in plasma and bile samples

A tracer amount of [^{125}I]hEGF (40 μCi/kg body wt), dissolved in 300 μl of rat plasma, was administered intravenously, and the blood samples were withdrawn at designated times (0.5, 5, 10, 30, 60 and 120 min) after i.v. administration. Bile samples were also collected at designated time intervals (0–30, 30–60 and 60–120 min). These plasma and bile samples were applied on a Sephadex G-25 column (0.9 × 28 cm) equilibrated and eluted with 1 M acetic acid solution containing 0.1% Triton X-100 and 1 M urea at 4°, on which 2 ml of 0.1% BSA solution had been preeluted. The elution rate was 2 ml·cm^{-2}·hr^{-1}, and 450 μl was collected for each fraction.

Calculation of kinetic parameters in the bolus administration experiments

Plasma concentration data of [^{125}I]hEGF after i.v. and h.p.v. administration were fitted to the following three-exponential and bioexponential equations by use of the nonlinear iterative least squares method [16] respectively:

$$C_p = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} + C \cdot e^{-\gamma t} \quad (1)$$

$$C_P = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} \quad (1)'$$

where α, β and γ are the apparent first-order rate constants and A, B and C are the corresponding zero-time intercepts respectively.

The area under the plasma concentration time curve (AUC) was calculated by Eq. (2) for the i.v. and Eq. (2)′ for the h.p.v. administration respectively [17]:

$$AUC_{\text{iv}} = A/\alpha + B/\beta + C/\gamma \quad (2)$$

$$AUC_{\text{hpv}} = A/\alpha + B/\beta \quad (2)'$$

The total body clearance (CL_{tot}) and the steady-state distribution volume (Vd_{ss}) are calculated by Eqs. (3) and (4) respectively [17]:

$$CL_{\text{tot}} = \text{Dose}/AUC_{\text{iv}} \quad (3)$$

$$Vd_{\text{ss}} = \text{Dose} \cdot (A/\alpha^2 + B/\beta^2 + C/\gamma^2)/(AUC_{\text{iv}})^2 \quad (4)$$

The biliary excretion clearance during the time interval from 0 to 60 min (CL_{BL}) is given as follows [17]:

$$CL_{BL} = X_{BL}/AUC_{(0\text{–}60)} \quad (5)$$

where X_{BL} is the amount excreted into bile during the time interval from 0 to 60 min and $AUC_{(0\text{–}60)}$ is the area under the plasma concentration time curve from 0 to 60 min after i.v. or h.p.v. administration.

Calculation of kinetic parameters in infusion study

In infusion experiments, CL_{tot} is expressed as [17]:

$$CL_{\text{tot}} = I/C_A^{SS} \quad (6)$$

where I is the infusion rate and the C_A^{SS} is the arterial plasma concentration at steady state. If we assume that removal of hEGF in the gastrointestinal tract and other organs draining to the liver is negligible, the extraction ratio of the liver is given as follows [17]:

$$E_H = (C_A^{SS} - C_{HV}^{SS})/C_A^{SS} \quad (7)$$

The extraction ratio of the kidney is also given as follows [17]:

$$E_R = (C_A^{SS} - C_{RV}^{SS})/C_A^{SS} \quad (8)$$

where C_A^{SS}, C_{HV}^{SS} and C_{RV}^{SS} are the steady-state plasma concentrations in the artery, hepatic vein and renal vein respectively.

The hepatic clearance (CL_H) and the renal clearance based on the plasma concentration ($CL_{R,P}$) are defined as follows [17]:

$$CL_H = Q_{P,H} \cdot E_H \quad (9)$$

$$CL_{R,P} = Q_{P,R} \cdot E_R \quad (10)$$

where $Q_{P,H}$ and $Q_{P,R}$ are the plasma flow rates in the liver and the kidney respectively.

The biliary excretion clearance (CL_{BL}) and the renal clearance based on the urinary excretion ($CL_{R,U}$) are given as follows [17]:

$$CL_{BL} = \Delta X_B/\Delta t/C_A^{SS} \quad (11)$$

$$CL_{R,U} = \Delta X_U/\Delta t/C_A^{SS} \quad (12)$$

where ΔX_B and ΔX_U are the accumulated amounts excreted into the bile and the urine during time interval Δt at steady state.

RESULTS

The elution profiles of [^{125}I]hEGF in rat plasma on a Sephadex G-25 column are shown in Fig. 1. [^{125}I]hEGF was eluted in the void volume. The ratio of the area in the peak of [^{125}I]hEGF to the sum of the areas under both peaks decreases and that of the metabolites increase with time after i.v. administration. [^{125}I]hEGF was stable in plasma when determined *in vitro*, so the decrease in the fraction of [^{125}I]hEGF may represent the [^{125}I]hEGF metabolism in the body. The plasma and bile samples, which were examined by gel filtration, were also analyzed by the TCA precipitation method to compare the two methods. A good correlation between both methods suggested that the TCA precipitation method can be used in the determination of [^{125}I]hEGF instead of chromatographic analysis (Fig. 2). Therefore, we used the more simple TCA precipitation method in the following experiments.

The [^{125}I]hEGF plasma concentration time profiles after i.v. and h.p.v. administration of a tracer dose of [^{125}I]hEGF are shown in Fig. 3. There exists a remarkable administration-route dependency in the plasma concentration time profiles of [^{125}I]hEGF, particularly in the early distribution phase. In the

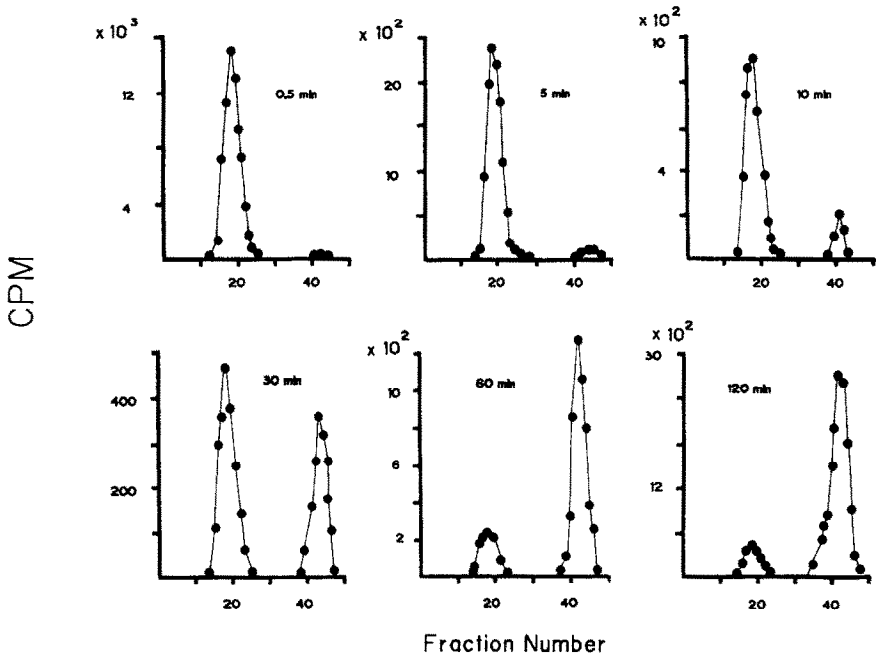

Fig. 1. Elution profiles of [^{125}I]hEGF in rat plasma at various sampling times after intravenous administration of a tracer dose of [^{125}I]hEGF. A 50-μl plasma sample was adjusted to 300 μl with the elution buffer and applied to a Sephadex G-25 column (0.9 × 28 cm). The volume of one fraction was 450 μl, and the elution rate was 2 ml·cm^{-2}·hr^{-1}. The ordinate is the total cpm of each fraction.

case of h.p.v. administration, [^{125}I]hEGF has to pass through the liver before appearing in the systemic circulation; such a great route dependency, therefore, suggests that the liver is the major organ in the early distribution of hEGF, confirming our previous observation [6].

The biliary excretion profiles of [^{125}I]hEGF after i.v. and h.p.v. administration are shown in Fig. 4. The accumulated amount of [^{125}I]hEGF into the bile during 60 min was 1–2% of the dose, and the lag time in the biliary excretion of hEGF was approximately 10 min in both cases.

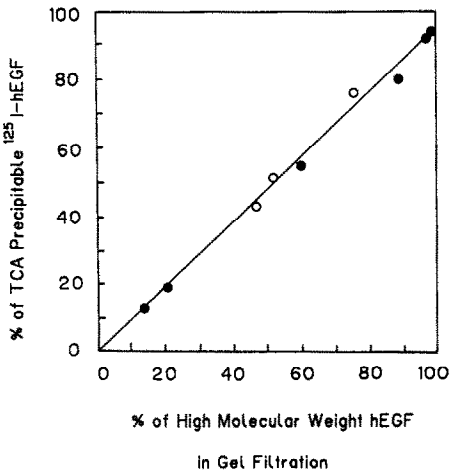

Fig. 2. Relationship between the TCA precipitable percent and the percent of radioactivity in the void volume in gel filtration. The plasma and bile samples from i.v. administration of [^{125}I]hEGF were divided into two fractions for the determination of TCA precipitable percent and the percent of radioactivity in the void volume by gel filtration. The TCA precipitable percent was calculated as (cpm in precipitate)/((cpm in precipitate) + (cpm in supernatant)) × 100. The percent of radioactivity in the void volume in gel filtration was calculated as (cpm in the void volume peak)/((cpm in the void volume peak) + (cpm in the latter peak)) × 100. Key: (○) bile, and (●) plasma.

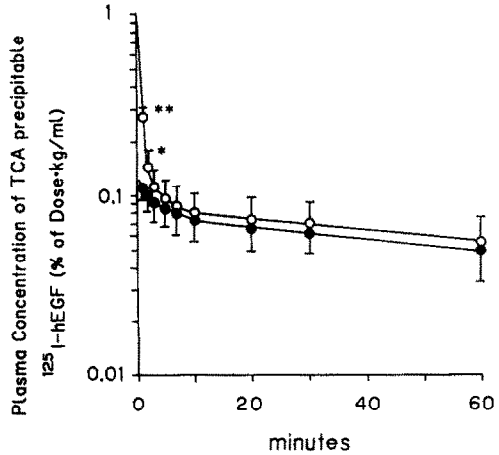

Fig. 3. Plasma concentration time profiles of [^{125}I]hEGF after i.v. or h.p.v. administration of tracer [^{125}I]hEGF in rats. Each point is the mean ± SE of five animals. Key: (○) i.v., and (●) h.p.v.; (**) $P < 0.01$, and (*) $P < 0.05$ (vs h.p.v. administration).

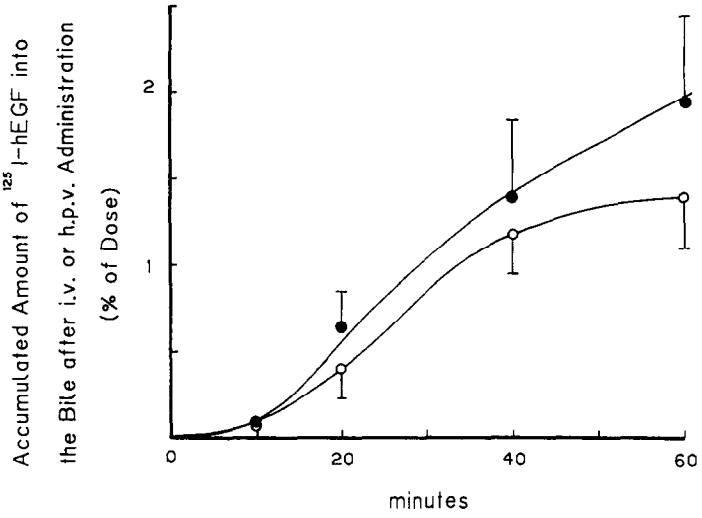

Fig. 4. Accumulated amount of [^{125}I]hEGF in the bile after i.v. or h.p.v. administration in rats. Key: (○) i.v., and (●) h.p.v. Each point is the mean ± SE of five animals.

The pharmacokinetic parameters in the bolus administration experiments are summarized in Table 1. We fitted the plasma concentration data of [^{125}I]hEGF after i.v. administration to the three-exponential equation since it gave smaller AIC values than those obtained by fitting the same data to the two-exponential equation. Regardless of a remarkable administration-route dependency in the plasma concentration time profiles (Fig. 3), it is not desirable to determine the E_H from the difference in $AUCs$ between the i.v. and h.p.v. administration, considering that a small variance in the estimation of the apparent slow disposition rate constants (γ) in Eq. (1) and that (β) in Eq. (1)′ may induce considerable errors in the estimation of $AUCs$ by Eq. (2) and (2)′. In addition, the parameters CL_{tot} and Vd_{ss} obtained from the i.v. bolus experiments may not give such precise values for the same reason. Therefore, we further attempted to determine the extraction ratio of hEGF in the liver and kidney by using the constant infusion technique.

The time courses of the arterial and hepatic vein plasma concentrations of [^{125}I]hEGF and [^{14}C]inulin during the constant intravenous infusion are shown in Fig. 5, and the calculated pharmacokinetic parameters are summarized in Table 2. The E_H of [^{125}I]hEGF determined from the plasma concen-

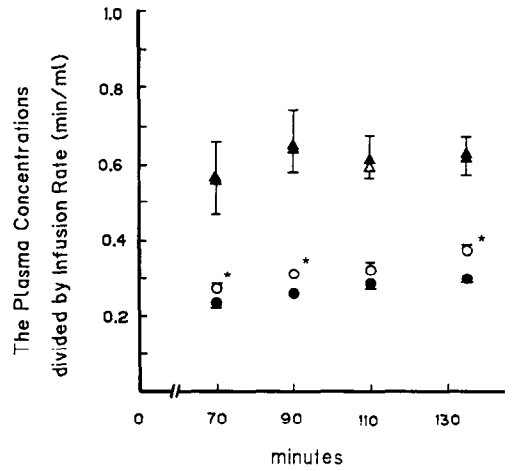

Fig. 5. Ratios of the plasma concentrations of [^{125}I]hEGF and [^{14}C]inulin (cpm/ml plasma) to each infusion rate (cpm/min/rat) during constant intravenous infusion. Key: (○) [^{125}I]hEGF in the artery plasma, (●) [^{125}I]hEGF in the hepatic vein plasma, (△) [^{14}C]inulin in the artery plasma, and (▲) [^{14}C]inulin in the hepatic vein plasma. Each point represents the mean ± SE of three animals. An asterisk (*) indicates $P < 0.05$ (vs the hepatic vein plasma).

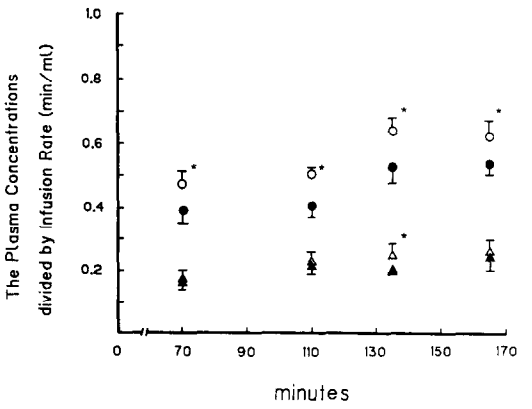

Fig. 6. Ratios of the plasma concentrations of [^{125}I]hEGF and [^{14}C]inulin (cpm/ml plasma) to the corresponding infusion rates (cpm/min/rat) of [^{125}I]hEGF and [^{14}C]inulin during constant intravenous infusion. Key: (○) [^{14}C]inulin in the artery plasma, (●) [^{14}C]inulin in the renal vein plasma, (△) [^{125}I]hEGF in the artery plasma, and (▲) [^{125}I]hEGF in the renal vein plasma. Each point is the mean ± SE of three animals. An asterisk (*) indicates $P < 0.05$ (vs the renal vein plasma).

Table 1. Pharmacokinetic parameters of [^{125}I]hEGF obtained from the plasma concentration time profiles after intravenous (i.v.) and hepatic portal vein (h.p.v.) administration of [^{125}I]hEGF in rats*

Route of administration	A† (% dose/ml/kg)	α† (min^{-1})	B† (% dose/ml/kg)	β† (min^{-1})	C† (% dose/ml/kg)	γ† (min^{-1})	AUC‡ (% dose·min/ml/kg)	CL_{tot}§ (ml/min/kg)	Vd_{ss}‖ (ml/kg)	CL_{BL}¶ (ml/min/kg)
Jugular vein	0.793 ± 0.133	1.66 ± 0.17	0.076 ± 0.027	0.328 ± 0.094	0.083 ± 0.027	0.004 ± 0.001	25.8 ± 10.6	7.06 ± 2.30	1669 ± 484	0.432 ± 0.114
Portal vein	0.054 ± 0.008	0.381 ± 0.046	0.079 ± 0.022	0.009 ± 0.001			13.1 ± 6.9			0.685 ± 0.203

* Each value is the mean ± SE of five animals.
† Calculated by fitting the plasma concentration data after i.v. or h.p.v. administration to Eq. (1) and (1)′ by use of the nonlinear iterative least squares method [16].
‡ The area under the plasma concentration time curve to the infinite time after i.v. or h.p.v. administration was calculated by Eq. (2) and (2)′.
§ The total body clearance was calculated by Eq. (3).
‖ The steady-state distribution volume of [^{125}I]hEGF was calculated by Eq. (4).
¶ The biliary excretion clearance of [^{125}I]hEGF after i.v. or h.p.v. administration was calculated by Eq. (5).

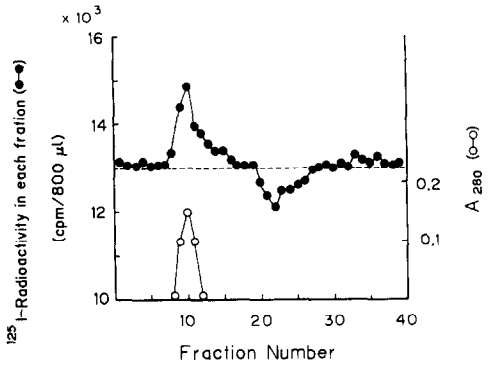

Fig. 7. Elution profiles of pooled rat plasma preincubated with [^{125}I]hEGF on a Sephadex G-75 column equilibrated with 0.13 M phosphate buffer containing [^{125}I]hEGF. Absorbance at 280 nm (A_{280}) and radioactivity were measured in each fraction (0.8 ml).

tration difference between the artery and the hepatic vein at steady state (after 110 min) was 0.19, while that of [^{14}C]inulin was negligible. Assuming that the hepatic plasma flow rate was 40 ml/min/kg body wt [18–21], the CL_H of [^{125}I]hEGF calculated by Eq. (9) was 7.56 ml/min/kg body wt. This value was approximately 70% of the CL_{tot} (10.8 ml/min/kg body wt). These results clearly indicate that the liver was the major organ determining the systemic [^{125}I]hEGF plasma concentration at steady state.

Figure 6 shows [^{125}I]hEGF concentrations in the artery and renal vein after performing similar infusion experiments. The calculated pharmacokinetic parameters are summarized in Table 3. The E_R for [^{14}C]inulin (0.18) is comparable to that reported by others [22], suggesting that the kidney had normal glomerular filtration function during the infusion experiments. The E_R of [^{125}I]hEGF obtained based on the plasma concentration (0.1) was approximately half that of [^{14}C]inulin, which was eliminated only by glomerular filtration from the kidney.

To investigate the lower renal clearance of [^{125}I]hEGF compared to that of [^{14}C]inulin, the plasma protein binding of [^{125}I]hEGF was determined by charcoal adsorption and equilibrium gel-filtration methods (Fig. 7). The plasma protein bound percentages of [^{125}I]hEGF determined by the former and the latter methods were 51.2 ± 2.9 (tracer, 10, 100 and 1000 nM) and 54.2% (tracer only) respectively. Thus, either method gave comparable unbound fractions.

DISCUSSION

In a previous paper [6], we reported that the liver and kidney are the main organs responsible for the distribution of hEGF in the early distribution phase (<5 min). We also showed the possibility of receptor-mediated binding (or uptake) to these organs based on *in vivo* studies. In the present study, we further determined the metabolic and/or excretory clearance of hEGF in the liver and kidney based on plasma data and also examined biliary and urinary

Table 2. Pharmacokinetic parameters of [^{125}I]hEGF and [^{14}C]inulin calculated from the plasma concentration and biliary excretion profiles during constant infusion in rats*

	CL_{tot}† (ml/min/kg)	E_H‡	CL_H§ (ml/min/kg)	CL_{BL}‖ (ml/min/kg)
[^{14}C]Inulin	6.35 ± 0.82	UD¶	UD	0.063 ± 0.024
[^{125}I]hEGF	10.8 ± 0.7	0.189 ± 0.003	7.56 ± 1.27	0.227 ± 0.036

* Each value is the mean ± SE of three animals.
† The total body clearance was calculated by Eq. (6).
‡ The hepatic extraction ratio at steady state was calculated by Eq. (7). The plasma concentrations at 110 and 135 min after the initiation of infusion were used in the calculation.
§ The hepatic clearance was calculated by Eq. (9) using the reported value (40 ml/min/kg body wt) of hepatic plasma flow rate [18–21].
‖ The biliary excretion clearance was calculated by Eq. (11).
¶ Undetectable (the difference in the concentrations between the artery and the hepatic vein was too small).

Table 3. Pharmacokinetic parameters of [^{125}I]hEGF and [^{14}C]inulin calculated from the plasma concentration and urinary excretion profiles during constant infusion in rats*

	CL_{tot}† (ml/min/kg)	E_R‡	$CL_{R,P}$§ (ml/min/kg)	$CL_{R,U}$‖ (ml/min/kg)
[^{14}C]Inulin	6.24 ± 0.38	0.175 ± 0.028	4.73 ± 0.73	6.96 ± 1.59
[^{125}I]hEGF	15.6 ± 1.5	0.098 ± 0.030	2.65 ± 0.72	0.291 ± 0.068

* Each value is the mean ± SE of three animals.
† The total body clearance was calculated by Eq. (6).
‡ The renal extraction ratio at steady state was calculated by Eq. (8). The plasma concentrations at 110 and 135 min after the initiation of infusion were used in the calculation.
§ The renal clearance based on the plasma concentration was calculated by Eq. (10) using the reported value (27 ml/min/kg) for renal plasma flow [21–23].
‖ The renal clearance based on the urinary excretion was calculated by Eq. (12).

excretion clearances of hEGF by measuring biliary and urinary excretion.

Matrisian *et al.* [24] reported that EGF, iodinated by the chloramine-T method, is heterogeneous and revealed different binding behaviors to EGF receptor, but the heterogeneous [^{125}I]EGFs had equal potencies in stimulating DNA synthesis regardless of their different binding behaviors. [^{125}I]hEGF used in our experiment was displaced from its specific receptor by the addition of unlabeled hEGF in both rat liver homogenates and in isolated rat hepatocytes [9], and this [^{125}I]hEGF was also displaced from the liver, kidney and small intestine, known to have specific receptors for EGF on the cell surface membrane, by coadministration of an excess amount of unlabeled hEGF *in vivo* [6]. Therefore, [^{125}I]hEGF used in our experiment competed for the binding to its specific receptor with unlabeled hEGF both *in vitro* and *in vivo*. Furthermore, the hEGF iodinated by a milder lactoperoxidase method [25] had comparable binding activity in liver homogenates with the hEGF iodinated by the chloramine-T method (unpublished observation). With these findings in mind, it may be reasonable to assume that the pharmacokinetic behavior of [^{125}I]hEGF used in the present study is comparable to that of the unlabeled hEGF.

The CL_H obtained by multiplying E_H at steady state by hepatic plasma flow rate was approximately 70% of the CL_{tot} (Table 2). Therefore, the liver is considered to be the main organ in the control of EGF plasma concentration. In the calculations of hepatic and renal clearances of hEGF, however, we used plasma flow rates based on the literature values. The reported values of the hepatic and renal plasma flow rates were 40 ± 6 ml/min/kg body wt [18–21] and 27 ± 3 ml/min/kg body wt [21–23]. Also in our laboratory, we previously measured the hepatic and renal plasma flow rates, and these values (liver: 35 ml/min/kg body wt [12], kidney: 29 ml/min/kg body wt [26]) fall within the literature values. Considering these facts, the plasma flow rates used in the present calculation may be appropriate.

The value of CL_{tot} (10.8 ± 0.7 ml/min/kg body wt) of [^{125}I]hEGF in Table 2 was somewhat different from that (15.6 ± 1.5 ml/min/kg body wt) of [^{125}I]hEGF in Table 3. Although the reason for this discrepancy remains to be resolved, it may be due to the difference in the position of cannulation between the two methods. That is, in the former experiments we cannulated the femoral artery, femoral vein, jugular vein, hepatic vein and bile duct, and in the latter experiments we cannulated the femoral artery, femoral vein, jugular vein, renal vein and ureters.

The values for CL_{BL} of hEGF determined in the i.v. bolus administration and infusion experiments were 0.43 and 0.23 ml/min/kg body wt, respectively, which is 3–6% of the CL_H (Tables 1 and 2). Therefore, the major pathway in the hepatic processing of

hEGF is considered to be the metabolism through the receptor-mediated cellular uptake followed by the lysosomal degradation [2].

Several kinds of evidence have suggested the presence of dual pathways for translocation of EGF across hepatocytes from plasma into the bile [27]: a direct vesicular pathway from the sinusoidal membrane to the bile canaliculus and an indirect pathway involving lysosomal degradation [28]. In the direct pathway, the endocytotic vesicle is considered to serve as a direct shuttle vesicle, forming at the sinusoidal surface, traversing the cell to the peribiliary space, fusing with the bile canalicular membranes, and releasing its contents into the bile by exocytosis [27]. We previously reported that the specific binding of [^{125}I]hEGF to the bile canalicular membrane vesicles is approximately half that for the sinusoidal membrane vesicles [29]. In addition, there exists a lag time of approximately 10 min for the [^{125}I]hEGF to appear in the bile (Fig. 4). The transit time through the bile ducts and catheter may be approximately 2 min, considering the catheter volume (7.4 μl, i.d. 0.28 mm × 12 cm), the volume (26 μl) of the bile duct in rats [30], and the bile flow rates (14–19 μl/min/rat) in our experiments. Therefore, the time required for the bile flow through the ducts to the sampling point cannot account for the lag time (~10 min) in biliary excretion of [^{125}I]hEGF. From these findings, the lag time in the biliary excretion of [^{125}I]hEGF (Fig. 4) is considered to represent the processing of hEGF from the sinusoidal membrane to the bile. However, the physiological meaning of the presence of EGF in the bile and the transport mechanism of EGF from the sinusoidal membrane to the bile have not been made clear.

When we define the renal clearance as the ratio of elimination rate from the plasma by the kidney to the drug concentration in the renal arterial plasma, the renal clearance ($CL_{R,P}$) can be described by the following equation [31]:

$$CL_{R,P} = (CL_{RF} + CL_{RS}) \cdot (1 - FR) \quad (13)$$

where CL_{RF} is renal filtration clearance, CL_{RS} is renal secretion clearance, and FR is the fraction of drug filtered and/or secreted that is reabsorbed.

When we define the renal clearance as the ratio of the urinary elimination rate to the drug concentration in the renal arterial plasma, the renal clearance can be described as follows:

$$CL_{R,U} = (CL_{RF} + CL_{RS}) \cdot (1 - FR - M) \quad (14)$$

where M is a dimensionless constant equal to the fraction of filtered and/or secreted drug that is metabolized in the renal tubules. Implied in the equation is the assumption that FR and M for the filtered and secreted drug are the same and that the metabolism of the drug in the kidney does not occur in the basolateral side of the renal tubules. If the metabolism of drugs that are filtered and/or secreted into the renal tubules occurs, as with many low molecular weight peptides or proteins, the renal clearance based on the urinary excretion ($CL_{R,U}$) would always be smaller than the renal clearance based on the plasma concentration ($CL_{R,P}$), because $CL_{R,P}$ would not be affected by the metabolism in the renal tubules.

The $CL_{R,U}$ of [^{125}I]hEGF, which is calculated from the amount excreted into the urine by Eq. (12), is approximately 11% of the $CL_{R,P}$ (Table 3). From these findings, it could be considered that the bulk of [^{125}I]hEGF cleared from the plasma by the kidney is metabolized further in the renal tubules before appearing in the urine. The $CL_{R,P}$ of [^{125}I]hEGF was approximately half of the CL_R of [^{14}C]inulin (Table 3). This lower value of $CL_{R,P}$ of [^{125}I]hEGF compared to that of [^{14}C]inulin may be explained by the lower plasma unbound fraction of [^{125}I]hEGF (approximately 0.5) (Fig. 7). If we assume the renal plasma flow rate is 27 ml/min/kg body wt, the $CL_{R,P}$ obtained by multiplying the plasma flow by E_R is 2.7 ml/min/kg body wt, and this value is approximately 17% of the CL_{tot} of [^{125}I]hEGF. Although the contribution of $CL_{R,P}$ to the CL_{tot} was thus smaller than that of the CL_H, the sum of CL_H and $CL_{R,P}$ was almost 90% of the CL_{tot}.

In conclusion, the liver and kidney were the major organs responsible for the elimination and/or metabolism of hEGF from the plasma, and the contributions of the liver and kidney to the total body clearance were approximately 70 and 20% respectively.

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