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Down-regulation and its effect of epidermal growth factor receptors on the pharmacokinetics of human epidermal growth factor after i.v. administration in rats

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Summary

Down-regulation of epidermal growth factor (EGF) receptors and subsequent restoration of down-regulated EGF receptors were investigated in rats. Pharmacokinetic behavior of human EGF (hEGF) after EGF receptors were down-regulated was also studied. Down-regulation of EGF receptors was induced by a bolus i.v. administration of hEGF at a dose of 500 μg/kg (pretreated rats). The pretreated rats received again hEGF i.v. (100 μg/kg) 3, 6, 24, 48, 72, 96, or 120 h after pretreatment and the plasma concentrations of immunoreactive hEGF were periodically determined after the second administration. Higher plasma concentrations of hEGF were observed after pretreatment. A greater AUC value of hEGF, 6–7 times that in untreated rats, was observed at 6 and 48 h after pretreatment and thereafter the increased AUC value declined gradually towards the control level of untreated rats. Time-dependent changes in total plasma clearance (CL_{total}) of hEGF after pretreatment were considered to be derived mainly from changes in the distribution volume (V_c) of hEGF in the central compartment. Changes of accessible EGF receptors after pretreatment were evaluated by specific binding of ^{125}I-EGF to the liver homogenates, hepatocytes and hepatocyte homogenates. A maximal decrease in the specific binding, 50% decrease of untreated rat, was observed in all specimens 6 h after pretreatment. Recovery of the binding in the liver homogenates and/or hepatocyte homogenates, probably due to the recycling and new synthesis of EGF receptors, commenced 6 h after pretreatment, and almost complete restoration was observed 120 h after pretreatment. On the other hand, in hepatocytes (intact cell), the steady-state of down-regulation of EGF receptors continued up to 72 h after pretreatment. No complete restoration of the specific binding to the hepatocytes was observed even 120 h after pretreatment and remained 30% lower than in untreated rats. A linear relationship between the CL_{total} value and the extent of down-regulation of EGF receptors in the hepatocytes was observed. Above results indicate that marked changes of pharmacokinetics of hEGF in pretreated rats result from the changes of accessible EGF receptors on the cell surfaces after pretreatment.

Introduction

Many biologically important peptides or proteins that bind to receptors on the cell surfaces such as low density lipoprotein (Brown and Gold-

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stein, 1979), insulin (Carpenter et al., 1979; Hachiya et al., 1987) and glucagon (Barazzone et al., 1980) are taken up by cells from extracellular fluid by receptor-mediated endocytosis as reviewed by Goldstein et al. (1979) and Wileman et al. (1985). Human epidermal growth factor (hEGF) or mouse epidermal growth factor (mEGF) is also taken up by the endocytic pathway in various cells such as human fibroblasts (Carpenter and Cohen, 1976), A-431 human epithelioid carcinoma cells (McKanna et al., 1979; Cohen and Fava, 1985), KB cells (Beguinot et al., 1984), 3T3 cells (Aharonov et al., 1978) and rat hepatocytes (Dunn and Hubbard, 1984). Extracellular EGF firstly binds to EGF receptors on the cell surface, a 170,000–180,000 molecular weight glycoprotein (Hunter, 1984), then the EGF-receptor complexes cluster in coated pits and are internalized to form receptosomes (Beguinot et al., 1984). The internalized EGF and receptors are degraded at lysosomes. Some of the internalized receptors cycle on the cell surface depending on the cell types and the physiological states (Beguinot et al., 1984; Dunn and Hubbard, 1984; Teslenko et al., 1987). The down-regulation of EGF receptors is believed to result from the internalization of the receptors and its subsequent intracellular degradation. As stated above, the endocytic pathway of EGF and EGF receptors and the resultant down-regulation of EGF receptors have been well recognized in various types of cells in vitro.

In an in vivo study employing rats, the endocytic internalization of EGF-receptor complexes and the resultant down-regulation of EGF receptors are also reported. Kay et al. (1986) reported that administration of 100 μg of EGF via the portal vein of rats resulted in 60% loss of EGF receptor from the liver plasmalemma as evaluated by ^{125}I-EGF binding.

In the previous report (Murakami et al., 1988), we demonstrated the extraordinary dose-dependent pharmacokinetic behavior of immunoreactive hEGF after i.v. administration in rats. The dose-dependent pharmacokinetics of hEGF were proved to be not due to the saturation of excretion processes such as biliary and urinary excretions (Murakami et al., 1989) but due to the binding saturation to receptors as reported by Yanai et al. (1987) and Kim et al. (1988).

In rats having down-regulated EGF receptors, the pharmacokinetic behavior of hEGF administered thereafter may be markedly influenced. In the present study, therefore, the effect of the down-regulation of EGF receptors on the pharmacokinetic behavior of hEGF after i.v. administration was investigated in rats.

Materials and Methods

Materials

hEGF, mol. wt. 6216, used in the present study was produced by Wakunaga Pharm. Co. Ltd. with genetic technology (Oka et al., 1985). ^{125}I-EGF used for the specific binding study in vitro was purchased from the Radiochemical Center (Amersham Corp., Arlington Heights, IL), bovine serum albumin (fraction V) from Sigma Chemical Co., (St. Louis, MO), collagenase S-1 from Nitta Gelatin Co., Ltd. Seibutukagaku Laboratories (Osaka Japan), and Medium 199 from Nissui Pharm. Co., Ltd. (Tokyo, Japan). Reagents used for the determination of hEGF by enzyme immunoassay were the same as reported previously (Murakami et al., 1988). All other reagents were of analytical grade and were used without further purification.

Animal study

Male Sprague–Dawley rats weighing 180–200 g were used. hEGF was dissolved in an isotonic pH 7.4, 0.05 M Tris-HCl buffer solution containing Tween 80 (0.01%) at a concentration of 500 μg/ml. The solution was administered i.v. via the rat tail vein at a dosing volume of 1 ml/kg to induce a down-regulation of EGF receptors (pretreated rats). The pretreated rats were housed under usual conditions (12 h light/12 h dark cycle) until the second i.v. administration of hEGF. Just prior to the second administration of hEGF, rats were anesthetized by an i.p. injection of sodium pentobarbital (Nembutal solution, Abbott Laboratories) at a dose of 30 mg/kg and were kept supine on a surface controlled at 37°C to maintain their body

temperature above 36 °C during experimental periods. The second i.v. administration of hEGF via the tail vein of the pretreated rats was performed at a dose of 100 μg/ml/kg 3, 6, 24, 48, 72, 96, or 120 h after the initial administration (pretreatment). Blood was collected from a jugular vein at appropriate time intervals and immediately centrifuged to obtain plasma samples. Plasma samples were stored at −30 °C until analyzed.

Determination of the extent of the down-regulation of EGF receptors

Extent of the down-regulation of EGF receptors was determined by ^{125}I-hEGF specific binding assay using rat liver homogenates, hepatocytes and hepatocyte homogenates. A rapid filtration technique (Dunn and Hubbard, 1984; Yanai et al., 1987) was used in the binding assay. The liver of pretreated rats was removed 3, 6, 24, 72, or 120 h after the i.v. administration of hEGF at a dose of 500 μg/kg. The liver was homogenized by a glass homogenizer in ice-cold water with 9-fold volume of pH 7.4 binding medium (Medium 199 containing 0.1% bovine serum albumin), which was oxygenated in advance by bubbling with 95% O_2 and 5% CO_2. To 350 μl of the liver homogenates, 100 μl of binding medium alone or binding medium containing unlabeled hEGF (10 μg/ml) was added and the mixture was preincubated at 4 °C for 30 min. After preincubation, 50 μl of ^{125}I-hEGF solution prepared with binding medium (0.2 μCi/ml) was added to the mixture. After 1.5 h incubation at 4 °C, 100 μl of the reaction mixture was filtered through a glass microfiber filter (Whatman Ltd., Maidstone, England) followed by washing with 5 ml of ice-cold binding medium to remove unbound ligands. Radioactivity of bound ligand on the filter was measured with an Auto Well Gamma System (ARC-600, Aloka Co., Ltd., Tokyo, Japan). Non-specific binding, as determined by measuring the binding in the presence of excess unlabeled hEGF (2 μg/ml), was less than 10% of the total binding. All experiments were performed in triplicate.

In a separate experiment, binding experiments were performed using hepatocytes and hepatocyte homogenates. Hepatocytes of pretreated rat were isolated at the designated time after pretreatment in the same manner as reported by Berry and Friend (1969) except for using binding medium as a recirculating perfusing medium and an incubation buffer. Perfusion solution containing 0.025% collagenase was recirculated for 12 min at 37 °C. Isolated cells were filtered through two layers nylon mesh (625–900 mesh/cm^2) and washed with the binding buffer 3 times. Isolated cells used in the present study were the mixture of parenchymal and non-parenchymal cells. Cells were resuspended in the fresh binding medium at a concentration of $1–5 \times 10^6$ cells/ml. The cell viability was determined by a Trypan blue exclusion test and cells showing more than 90% viability were used for the binding test. Hepatocyte homogenates were prepared by homogenizing the cell suspension. The binding procedures of ^{125}I-EGF to hepatocytes and hepatocyte homogenates were the same as for liver homogenates described above. The specific and non-specific binding of ^{125}I-EGF were normalized with protein contents.

Analytical methods

Plasma samples were diluted appropriately, but at least more than 10-fold, with a 0.01 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 0.1% NaN_3, 0.1% bovine serum albumin, 0.005% Tween 20 and 1 mM $MgCl_2$. The concentration of immunoreactive hEGF in plasma was determined by enzyme immunoassay for hEGF as reported previously (Murakami et al., 1988). The concentration of protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard protein.

Results and Discussion

Elimination of hEGF from plasma in rats of down-regulated EGF receptors

Down-regulation of EGF receptors was induced by i.v. administration of hEGF to rats at a dose of 500 μg/kg (pretreated rat). As reported previously (Murakami et al., 1988), with a stepwise increase in an i.v. dose of hEGF in a range from 50–1000 μg/kg, a marked dose-dependent decrease in the total plasma clearance (CL_{total}) of

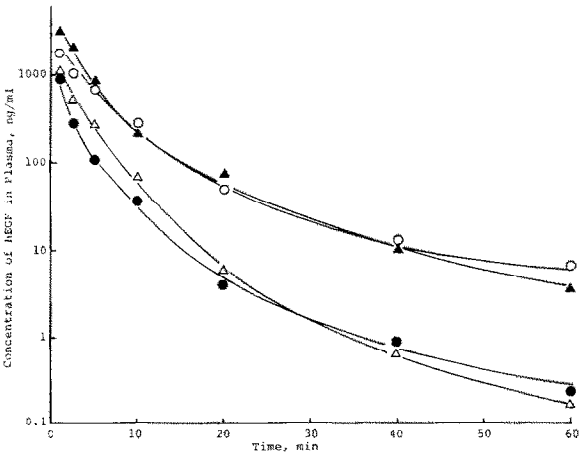

Fig. 1. Plasma concentrations of hEGF after i.v. administration at a dose of 100 μg/kg in untreated and pretreated rats. Pretreatment: 500 μg/kg hEGF was administered i.v. in advance, see text for details. ●——●, untreated rats; ▲——▲, ○——○ and △——△, pretreated rats (24, 72 and 120 h after pretreatment, respectively). Each point represents the mean with $n = 3$–5.

immunoreactive hEGF was observed. However, not much difference in the CL_{total} of immunoreactive hEGF was observed between the doses of 500 and 1000 μg/kg. The dose-dependent decrease in the CL_{total} was considered to be due to the saturation of EGF binding to receptors located on the cell surfaces of various organs. Thus, the dose of 500 μg/kg hEGF was employed to induce the down-regulation of EGF receptors. After 500 μg/kg hEGF was administered i.v., hEGF disappeared from plasma in a biexponential fashion with half-lives of 1.03 and 18.46 min for the α-phase and β-phase, respectively and became undetectable after 3 h (Murakami et al., 1988).

The pretreated rats received hEGF again i.v. at a dose of 100 μg/kg 3, 6, 24, 48, 72, 96, or 120 h after initial administration for pretreatment, and the plasma concentrations of immunoreactive hEGF after each second administration were periodically determined. As typical examples, the results obtained 24, 72 and 120 h after pretreatment are shown in Fig. 1 with the result of untreated rats.

Immunoreactive hEGF concentrations in plasma declined in a biexponential fashion in all

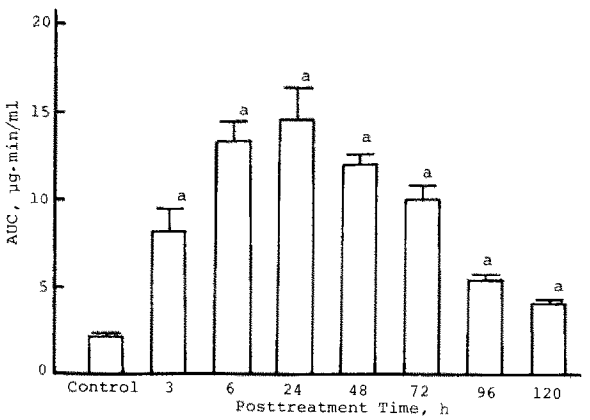

Fig. 2. AUC value of hEGF administered i.v. at a dose of 100 μg/kg in untreated and pretreated rats. Pretreatment: 500 μg/kg hEGF was administered i.v. in advance, see text for details. The error bars represent the S.E.M. with $n = 3$–5.
[a] Significantly different from control ($P < 0.05$).

cases and was described by the following equation;

$$C = Ae^{-\alpha t} + Be^{-\beta t}$$

The fitting analysis was performed by use of the Multi-Range program (Yamaoka et al., 1983). Changes of the area under the plasma concentration vs time curves (AUC) of hEGF, calculated by $A/\alpha + B/\beta$, in pretreated rats are shown in Fig. 2.

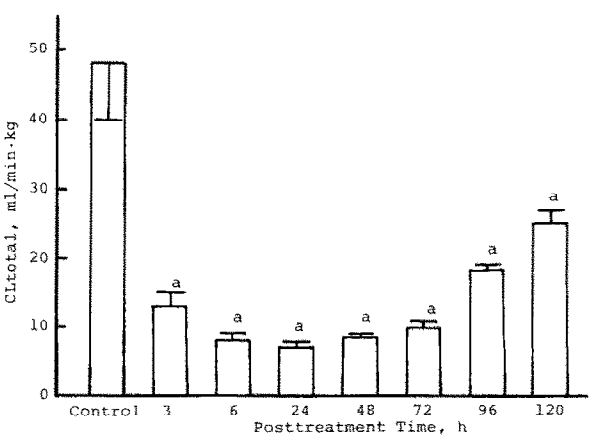

Fig. 3. CL_{total} values of hEGF administered i.v. at a dose of 100 μg/kg in untreated and pretreated rats. Pretreatment: 500 μg/kg hEGF was administered i.v. in advance, see text for details. The error bars represent the S.E.M. with $n = 3$–5.
[a] Significantly different from control ($P < 0.05$).

Significantly increased AUC values of hEGF compared with that of untreated rats were observed in all pretreated rats. Greater AUC value, 6–7 fold that in untreated rats, was observed during 6 and 48 h after pretreatment and thereafter the increased AUC value gradually returned to the level of untreated rats in a progress of time. The pharmacokinetic changes were also evaluated from a viewpoint of CL_{total}, calculated by dividing the dose of hEGF by AUC value, as shown in Fig. 3.

CL_{total} decreased rapidly after pretreatment and the values from 6 to 72 h were almost constant and then gradually restored towards the level of untreated rats. However, even at 120 h after pretreatment, value of CL_{total} was 52% of the untreated rats. The changes of CL_{total} after pretreatment will be accounted for by the changes of elimination rate constant (k_{el}) and/or distribution volume of central compartment (V_c), since the CL_{total} is expressed as a product of kel and V_c. Chronological changes of V_c, calculated by dividing the dose by ($A + B$), and k_{el} value, calculated by use of a two-compartment model, after pretreatment are shown in Fig. 4 and 5. The calculated V_c values changed almost in parallel with the changes of CL_{total}, whereas changes of k_{el} were

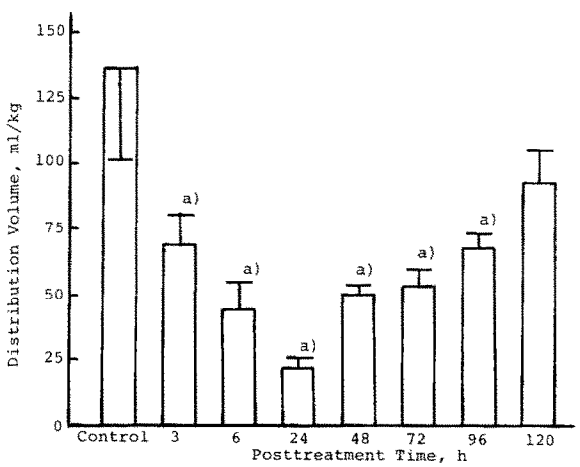

Fig. 4. V_c values of hEGF administered i.v. at a dose of 100 μg/kg in untreated and pretreated rats. Pretreatment: 500 μg/kg hEGF was administered i.v. in advance, see text for details. The error bars represent the S.E.M. with $n = 3–5$.
[a] Significantly different from control ($P < 0.05$).

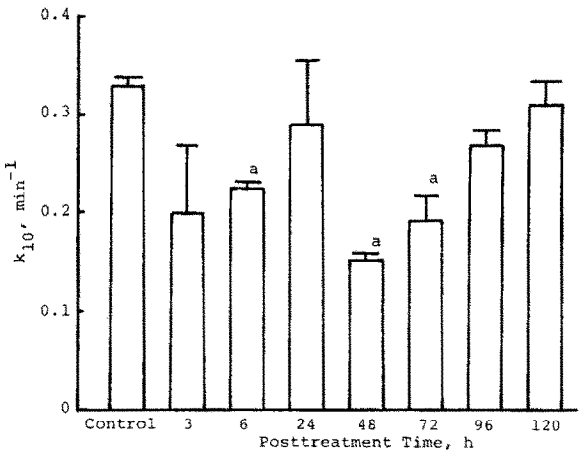

Fig. 5. The k_{el} values of hEGF administered i.v. at a dose of 100 μg/kg in untreated and pretreated rats. Pretreatment: 500 μg/kg hEGF was administered i.v. in advance, see text for details. The error bars represent the S.E.M. with $n = 3–5$.
[a] Significantly different from control ($P < 0.05$).

relatively small. V_c values 24 h after pretreatment showed less than the volume of circulatory plasma (40 ml/kg). The reason is not clear, however, hEGF is known to cause complicated vascular actions such as contraction of rat ileocolic artery (Muramatsu et al., 1985) and falling in total peripheral vascular resistance and an increase in cardiac output (Scoggins et al., 1984). The complicated vascular actions of hEGF may contribute to the lower value of V_c, especially in rats having down-regulated EGF receptors. The maximal decrease in V_c value was 1/5 of the untreated rats. However, the time-dependent changes in k_{el} value were scattered among times after pretreatment and the decreasing ratio of the k_{el} value to the untreated value was smaller than that of the V_c value. From above results, it was concluded that the time-dependent changes of CL_{total} after pretreatment are mainly derived from the changes of V_c value after pretreatment.

Down-regulation and its subsequent restoration of EGF receptors in vivo

Intravenously administered EGF distributes to various tissues (Covelli et al., 1972; Kim et al., 1988). However, the disappearance of hEGF from systemic circulation is mainly accounted for by hepatic clearance both at low and high doses (Kim

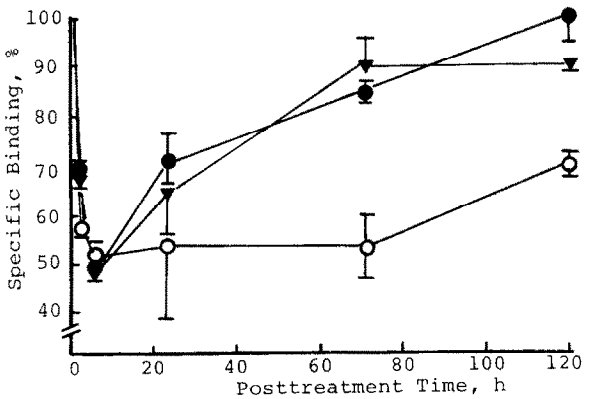

Fig. 6. Specific binding of ^{125}I-hEGF to liver homogenate (●———●), hepatocytes (○———○) and hepatocyte homogenates (▼———▼) of untreated and pretreated rats. Pretreatment: 500 μg/kg hEGF was administered i.v. in advance, see text for details. The error bars represent the S.E.M. with $n = 2$–3.

et al., 1988). The extent of the specific binding of hEGF to the liver is at least 10 times larger than those in other tissues (Yanai et al., 1987), and the hepatic uptake of hEGF is receptor-mediated endocytosis (Dunn and Hubbard, 1984). It is also known that EGF receptors distribute along the entire sinusoidal and lateral surfaces of hepatocytes under physiologically normal conditions, and upon the exposure of the cells to EGF, the EGF–receptor complexes are internalized and subsequently degradated at least partially at lysosomes (Dunn and Hubbard, 1984; Carpenter and Cohen, 1976; Haigler et al., 1979; Beguinot et al., 1984; Teslenko et al., 1987). Thus, the loss of accessible EGF receptors from the whole cells and the cell surfaces will be evaluated by determining the specific binding to the homogenates and intact cells, respectively.

The time-dependent changes of EGF receptors after pretreatment evaluated by the specific binding of ^{125}I-EGF to the liver homogenates, hepatocyte homogenates and hepatocytes (intact cells) are shown in Fig. 6. No significant difference in the specific binding was observed between the liver homogenates and hepatocyte homogenates. In the liver homogenates and/or hepatocyte homogenates, a maximal decrease in the specific binding, 50% decrease of the untreated rats, was observed 6 h after pretreatment and thereafter the binding was increased gradually towards the level of untreated rats. Almost complete restoration of the binding was observed 120 h after pretreatment. On the other hand, the decrease of the binding to the hepatocytes was larger than those of homogenates on and after 24 h. A marked difference in the extent of binding between the liver homogenates and hepatocytes (intact cells) 72 h and 120 h after pretreatment suggests possible intracellular existence of intact EGF receptors. Additionally, no complete restoration of EGF receptors was observed even 120 h after pretreatment (30% decrease of untreated rats). Thus, commencement of recovery of EGF receptors in liver homogenates was much faster, 6 h after pretreatment, than in hepatocytes. The restoration of specific binding in the homogenates may involve the recycling of receptors to the cell surfaces by dissociation of EGF receptor complexes and the de novo synthesis of EGF receptors after degradation. In contrast, the restoration of the binding to the hepatocytes seemed to commence at 72 h after pretreatment. The apparent time difference in the commencement of the restoration between the homogenates and hepatocytes may indicate the transit time of intracellular intact EGF receptors to the cell surfaces.

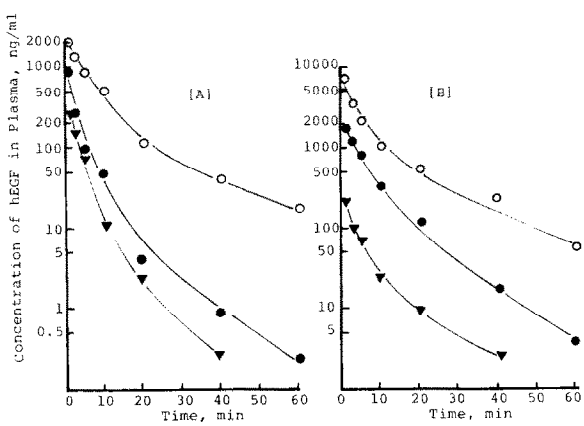

Fig. 7. Plasma concentration of hEGF after i.v. administration in untreated (A) and pretreated (B) rats. Pretreatment: 500 μg/kg was administered i.v. in advance. The second administration of hEGF was performed 6 h after pretreatment. Dose of hEGF: ▼———▼, 50 μg/kg; ●———●, 100 μg/kg; ○———○, 200 μg/kg. Each point represents the mean with $n = 3$–5.

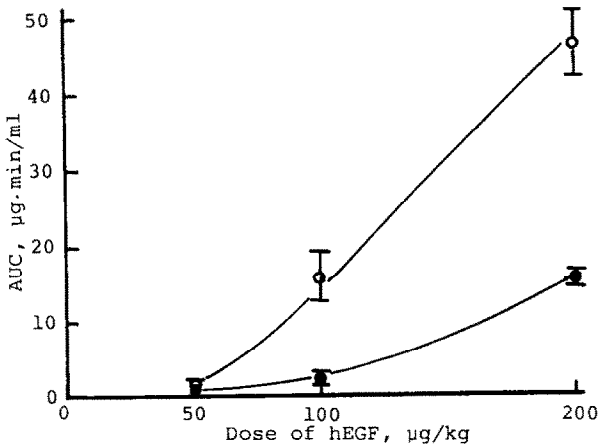

Fig. 8. Comparison of AUC value of hEGF administered i.v. between untreated and pretreated rats. Pretreatment: 500 μg/kg hEGF was administered i.v. in advance. The second administration of hEGF was performed 6 h after pretreatment, ●———●, untreated rats; ○———○, pretreated rats. Each point represents the mean and error bars represent S.E.M. with $n = 3$–5.

Dose-dependent pharmacokinetics of hEGF may be observed even in the pretreated rats, since accessible EGF receptors still remained by 50% of untreated rats on the cell surfaces of pretreated rats. The dose dependency of hEGF in pretreated rats was examined 6 h after pretreatment at 3 different doses. Plasma concentrations of immunoreactive hEGF were markedly increased in pretreated rats compared with those in untreated rats (Figs. 7 and 8). Although about 4-fold greater AUC values were observed in pretreated rats compared with those in untreated rats, a marked nonlinearity of pharmacokinetics was still observed (Fig. 8). Changes in the number of accessible EGF receptors distributed on the cell surfaces, or the extent of specific binding to the hepatocytes after pretreatment, may be actually responsible for changes of CL_{total} of hEGF after pretreatment. Thus, relationship between the extent of specific binding to hepatocytes and CL_{total} of hEGF observed at different times after pretreatment was examined (Fig. 9). A linear relationship was observed and it reveals that the time-dependent changes in pharmacokinetics of hEGF in the pretreated rats were due to the time-dependent negative modulations of EGF receptors on the cell surfaces after pretreatment. Further, based on these findings, the restoration of CL_{total} may represent the redistribution of intact EGF receptors on the cell surfaces after internalization.

The maximal down-regulation of EGF receptors or maximal changes in the pharmacokinetics of hEGF was observed 6 h and/or 24 h after pretreatment, whereas pretreated dose of hEGF (500 μg/kg) disappeared from plasma by 3 h. Carpenter and Cohen (1979) reported in their review that incubation of cells with subsaturating concentrations of EGF produced the down-regulation of unoccupied receptors, which may suggest cooperative interactions between occupied and unoccupied receptors. The time process for interactions between occupied and unoccupied receptors may account for the delayed action on EGF receptors after disappearance of pretreated dose of hEGF from plasma. However, the details of the process are still unclear.

The endocytic pathway of EGF receptor has been reported by many investigators as referred to in the text. The EGF receptors disappeared very rapidly (half-lives are less than 10 min) from the cell surface after exposuring to EGF in vitro (Carpenter and Cohen, 1976; Beguinot et al., 1984), in the perfused rat liver (Dunn and Hubbard, 1984), and also in vivo (Kay et al., 1986). How-

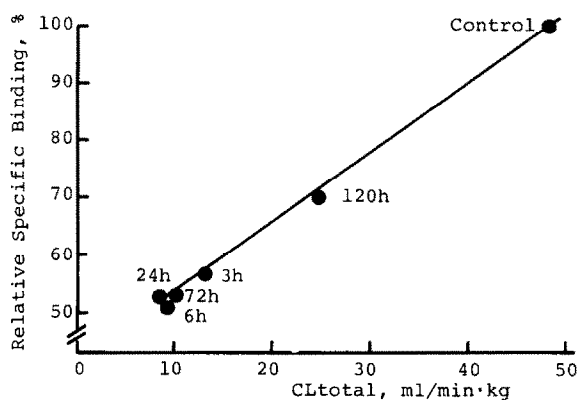

Fig. 9. Relationship between the extent of specific binding to hepatocytes and CL_{total} of hEGF administered i.v. at a dose of 100 μg/kg at different times after pretreatment. Pretreatment: 500 μg/kg hEGF was administered i.v. in advance. The figure next to the circle represents the time after pretreatment. Each point represents the mean with $n = 2$–5.

ever, in all cases reported, about 20–50% of initial EGF receptors remained on the cell surfaces as accessible receptors. The extent of remaining of EGF receptors on the cell surfaces after pretreatment was consistent with the present results. The internalized EGF–receptor complexes seem to exist as an intact form before lysosomal degradation. Dunn and Hubbard (1984) investigated EGF and receptor dynamics in the 4 h perfused rat liver and reported that no ^{125}I-EGF degradation was detected for at least 20 min after the internalization. They also reported that the rate of EGF clearance in the cell equaled the rate of ligand degradation and a constant pool of undegraded ligand remained in endosomes. During such steady state in the cell, accessible receptors on the cell surfaces will be also constant. In the present study, the maximal decrease of EGF receptors on the cell surfaces evaluated by specific binding in the hepatocytes was observed 6 h after pretreatment but the decreased binding never reached zero and the level of the maximal decrease was maintained over 72 h after pretreatment. These findings suggest the existence of undegraded EGF receptors on the surface of hepatocytes during 72 h after pretreatment. It is reported that on exposing cells to mEGF, the reduction of ^{125}I-EGF binding was observed and this reduction resulted from a decrease in the number of available EGF receptors per cell without any changes in the affinity of the receptors for EGF (Aharonov et al., 1978). Although Scatchard analysis was not performed in the present study, the decrease in the binding to the hepatocytes after pretreatment was assumed to be due to the decrease in the number of accessible EGF receptors on the cell surfaces. In the present study, V_c value of hEGF administered in pretreated rats was smaller than that of untreated rats. The decrease in V_c value after pretreatment may suggest the decrease in the number of accessible EGF receptors, although no quantitative relationship was observed between them.

With respect to the recovery of EGF receptors on the cell surfaces, Carpenter and Cohen (1976) reported that approximately 10 h of incubation of fibroblasts in a serum-containing medium was required for the complete recovery of the initial hEGF binding capacity after exposing the fibroblasts to 1 μg/ml of mEGF and that synthesis of both protein and RNA was required for the recovery of mEGF binding capacity. For 3T3 cells, Aharonov et al. (1978) reported that when EGF was removed from the medium, the EGF receptor was quickly replenished and one-half of the down-regulated receptors restored within 13 h. The restoration of degraded EGF receptors and redistribution of intact EGF receptors onto cell surfaces observed in the present study required much longer time than the results by Carpenter and Cohen (1976). Those discrepancies may be due to the difference in the concentration (or dose) of EGF used and/or the difference of experimental systems performed. Also, the possibilities of damaging liver function due to the higher dose of hEGF than they used are considered to be ruled out because biochemical indices of liver function such as GOT and GPT values were not influenced by the administration of hEGF at a high dose such as 10 mg/kg or more in mice (preliminary experiment). The effect of the pretreated dose on the down-regulation and consequent restoration of EGF receptors is currently under investigation.

In conclusion, marked changes of hEGF pharmacokinetics were demonstrated in association with the changes of specific binding of EGF to the receptors on the cell surfaces after the exposure to pretreated hEGF. Further investigations will be necessary to clarify in detail the mechanism behind the pharmacokinetic changes of hEGF after pretreatment and may give a rational clue for the establishment of dosage schedule of peptide drugs which are taken up by tissues in a receptor-mediated endocytic process.

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