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PHARMACOKINETIC ASPECTS OF PEPTIDE DELIVERY AND TARGETING: IMPORTANCE OF RECEPTOR-MEDIATED ENDOCYTOSIS

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A remarkable feature of the disposition of polypeptide hormones is the contribution of specific binding sites (receptors) on the cell-surface to their clearance in the body. Receptor-mediated endocytosis (RME) is now well recognized as a polypeptide clearance system. In addition, drug targeting utilizing RME is expected to have promise as a drug delivery system (DDS) to carry some drug specifically into the target cell expressing receptors on its plasma membrane. Therefore, it is important to analyze the RME process kinetically, both to clarify the pharmacokinetics of polypeptide itself and to estimate the efficiency of drug targeting via polypeptide receptors. We have been studying the hepatic handling of epidermal growth factor (EGF) and hepatocyte growth factor (HGF) using perfused rat liver, isolated rat hepatocytes, and primary cultured rat hepatocytes. Kinetic analysis of hepatic clearance of EGF enabled us to determine the kinetic parameters for the construction of a kinetic model for RME. Based on such a kinetic model, we can discuss the contribution of each process in RME on the efficiency of drug targeting into the cell. Not only RME, but also a non-specific uptake

mechanism was found to contribute to hepatic HGF disposition. This finding was important piece of information in developing a DDS for HGF, aimed at decreasing such non-specific uptake, with the result of prolonged plasma residence time.

INTRODUCTION

Recently, many types of polypeptides which induce the differentiation and/or growth of a great variety of cells have been discovered. Additionally, specific binding sites (receptors) related to the expression of their biological activity have also been identified, and their function and localization have been clarified. Polypeptides bind their receptor and form a complex on the cell-surface, followed by the uptake (internalization) of the complex via receptor-mediated endocytosis (RME). RME contributes to the elimination of polypeptides from the circulating blood (1,2).

On the other hand, the selective delivery of drugs to specific target tissue or cells by use of tissue- or cell-specific localization of polypeptide receptors has been expected to show promise as a drug delivery system (DDS). In this case, drugs are once coupled to a "vector" such as a peptide ligand or anti-receptor antibody which can bind the receptor, and then administered to the body. The drug can be delivered either into the intracellular space or to the opposite side of the cell via RME or receptor-mediated transcytosis, respectively.

In this lecture, we will introduce the kinetic analysis of RME of epidermal growth factor (EGF). This analysis in turn enabled us to construct a kinetic model for RME. And then, we will discuss the efficiency of drug targeting by RME from the kinetic point of view. In addition, we will also show the contribution of both RME and a non-specific uptake mechanism other than RME to the disposition of hepatocyte growth factor (HGF).

RESULTS AND DISCUSSION

(1) Kinetic Analysis of Hepatic Handling of EGF

(1-1) Kinetic Analysis of the Interaction between EGF and Cell-surface Receptor (3-5)

To determine kinetic constants for the relatively rapid interaction between ligand and cell-surface receptor, the multiple indicator dilution (MID) method in the single-pass perfused liver system was used (3). This method is an effective technique to obtain kinetic parameters with the structural architecture of the liver maintained (3,6). In the MID experiment, both a test polypeptide (EGF) and extracellular reference compound (^{14}C-inulin), were administered as a bolus into the portal vein, and the outflow in hepatic vein was sampled in about 0.5-1 sec aliquots. The reference compound passes through the liver without any interaction with the cell-surface (FIGURE 1), while the test compound interacts with cell-surface receptors followed by the sequestration into the intracellular compartment, only the remainder being detected in the outflow (FIGURE 1). Therefore, we can determine the kinetic parameters representing the interaction between the test compound and cell-surface receptor when we simultaneously analyze the concentration-time profiles (dilution curve) of both compounds in the outflow based on an appropriate mathematical model (6). In fact, the dilution curve of tracer ^{125}I-EGF was quite different from that of ^{14}C-inulin, while an almost identical curve was obtained for each compound when an excess amount of unlabeled EGF was coadministered (FIGURE 2), indicating the saturation of the EGF binding to the liver cell-surface. We determined the $k_{on,app}$ (= k_{on} R_s / V_d), k_{off}, and k_s values separately by means of simultaneous analysis of dilution curves of both a tracer ^{125}I-EGF and ^{14}C-inulin, where k_{on}, k_{off}, k_s, and R_s are the association rate constant, dissociation rate constant, sequestration rate constant, and cell-surface

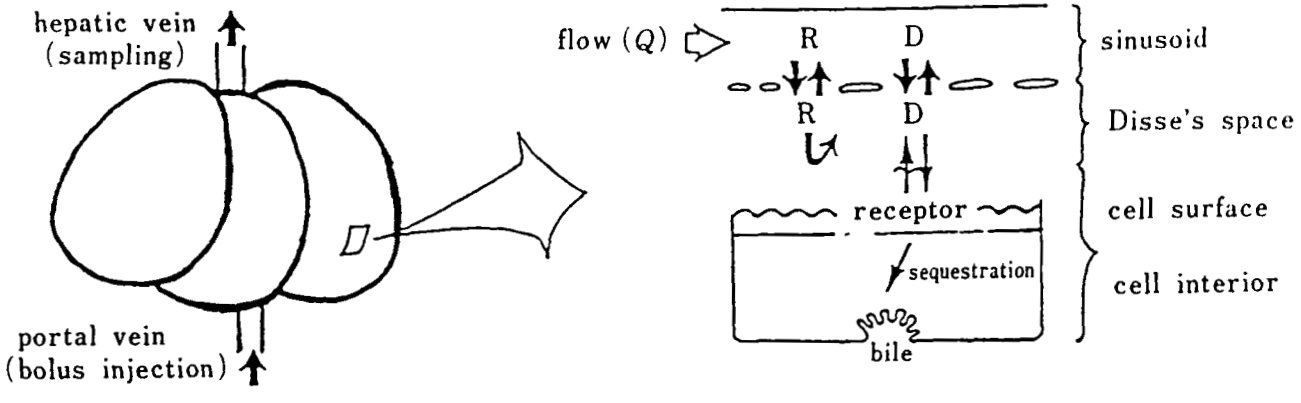

FIGURE 1

Schematic diagram for the multiple indicator dilution (MID) method

D, ligand in question, EGF etc.; R, extracellular reference, inulin etc.

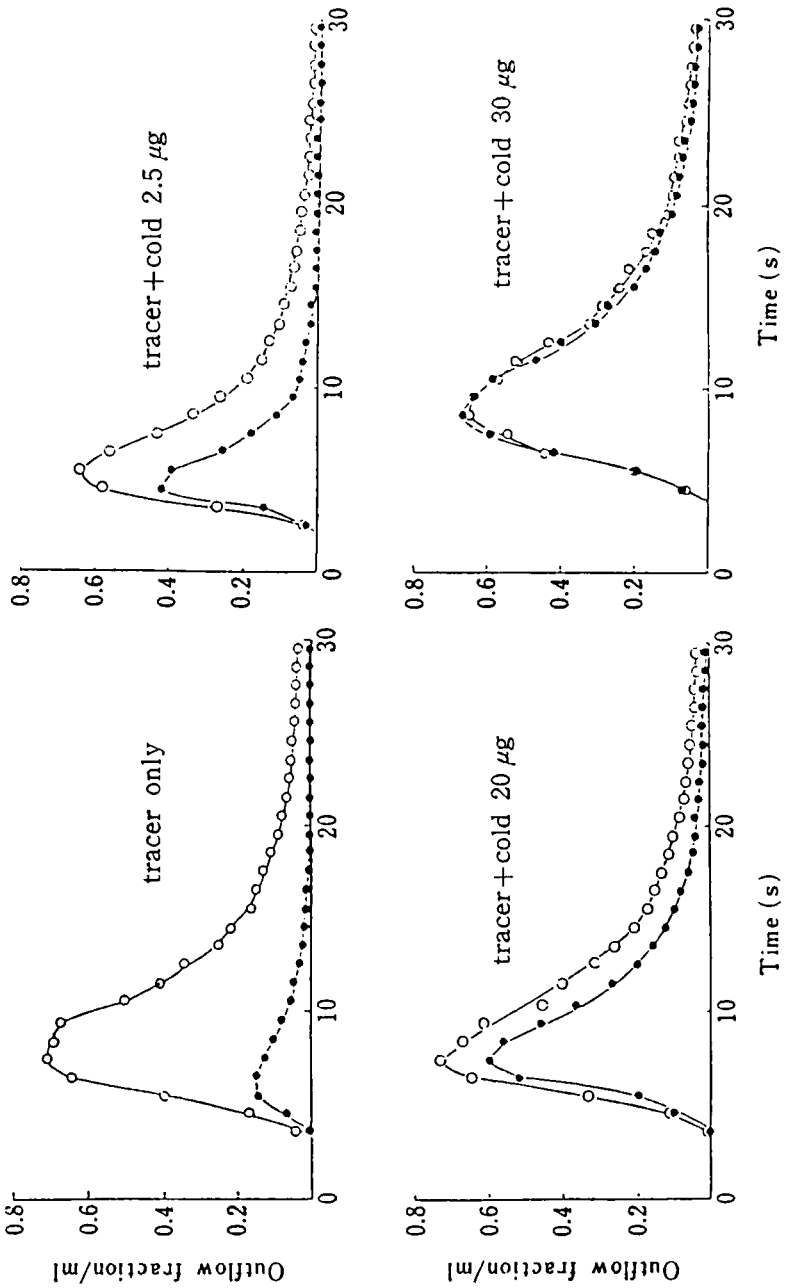

FIGURE 2
Dilution curves of ^{125}I-EGF (○) and ^{14}C-inulin (●) in the presence of various amounts of unlabeled EGF in the injectate (3).

TABLE 1
Parameters Representing the Receptor-mediated Endocytosis of EGF in the Liver.

Parameters*		Isolated hepatocytes		Perfused rat liver
		0° C	37° C	37° C
k_{on}	($min^{-1} \cdot nM^{-1}$)	0.07	0.7 - 1.1	0.12
k_{off}	(min^{-1})	0.15	1.1 - 1.6	2.1
Kd	(nM)	2.1	1.5	18
k_s	(min^{-1})		0.6 - 1.3	4.1
k_{int}	(min^{-1})		0.09 - 0.11	0.33
k_t	(min^{-1})		0.013 - 0.036	
$k_{deg,L}$	(min^{-1})		0.004 - 0.009	
$R_{s(t=0)}$	(pmol/g liver)	22	10 - 16	36
k_{-s}	(min^{-1})		0.01	
k_{ext}	(min^{-1})		0.015	
k_{bile}	(min^{-1})		0.0001	

*Abbreviations are shown in the legend of FIGURE 5.

unoccupied receptor density, respectively (3). The V_d is the extracellular volume which can be determined from the moment analysis of the dilution curve of ^{14}C-inulin. In addition, we obtained k_{on} and R_s separately by simultaneous analysis of the dilution curves of EGF at various doses (tracer - 30 μg)(3).

Also in isolated hepatocytes, the time-profiles of cell-associated EGF were analyzed kinetically to obtain the k_{on}, k_{off}, k_s, k_t, and R_s (Table 1), where k_t is internalization rate constant of unoccupied receptor (5).

Comparable values for each parameter, except the k_{on} value, were obtained for the two systems. That is, the k_{on}

value obtained from the perfused liver is approximately one order of magnitude smaller than that from the isolated hepatocytes. Although the reason for this discrepancy is not known, it can be explained when we consider that in the perfused liver system the association rate of EGF to the cell-surface receptor may be limited by the diffusion in the unstirred water layer which may exist in the interstitial space (Disse space) of the liver with its architecture maintained (7).

(1-2) Kinetic Analysis of the Internalization of EGF-receptor Complex(8-10)

After a tracer concentration of ^{125}I-EGF was perfused for selected times, both cell-surface bound ($[LR_s]$) and internalized ($[L_i]$) ^{125}I-EGF were measured separately using an acid-washing technique (FIGURE 3A)(8). The internalization rate is given by the following equation at elapsed times before intracellular degradation has occurred (4,13):

$$d[L_i]/dt = k_{int}[LR_s] \quad \text{Eq. (1)}$$

where k_{int} is the internalization rate constant, which represents the probability of internalization of the cell-surface EGF-receptor complex per unit time. Integration of Eq. (1) from time 0 to t yields:

$$[L_i] = k_{int} \int_0^t [LR_s]\, dt \quad \text{Eq. (2)}$$

The k_{int} value estimated from the initial slope of a plot of $[LR_i]$ vs $\int_0^t [LR_s]\, dt$ was 0.33 min^{-1} (FIGURE 3B)(8). This k_{int} value, however, was approximately 1/10 of the k_s value obtained from the MID method (TABLE 1). Also in isolated hepatocytes, the k_{int} value was much less than the k_s value (5). These results required two compartments (shallow and deep compartments) for the EGF-receptor complex on the cell-surface. Although the identification of these compartments is still awaited, one of the feasible explanations is that these compartments represent two different conformational states, and/or clustering of the complexes to a specific domain on the cell-surface.

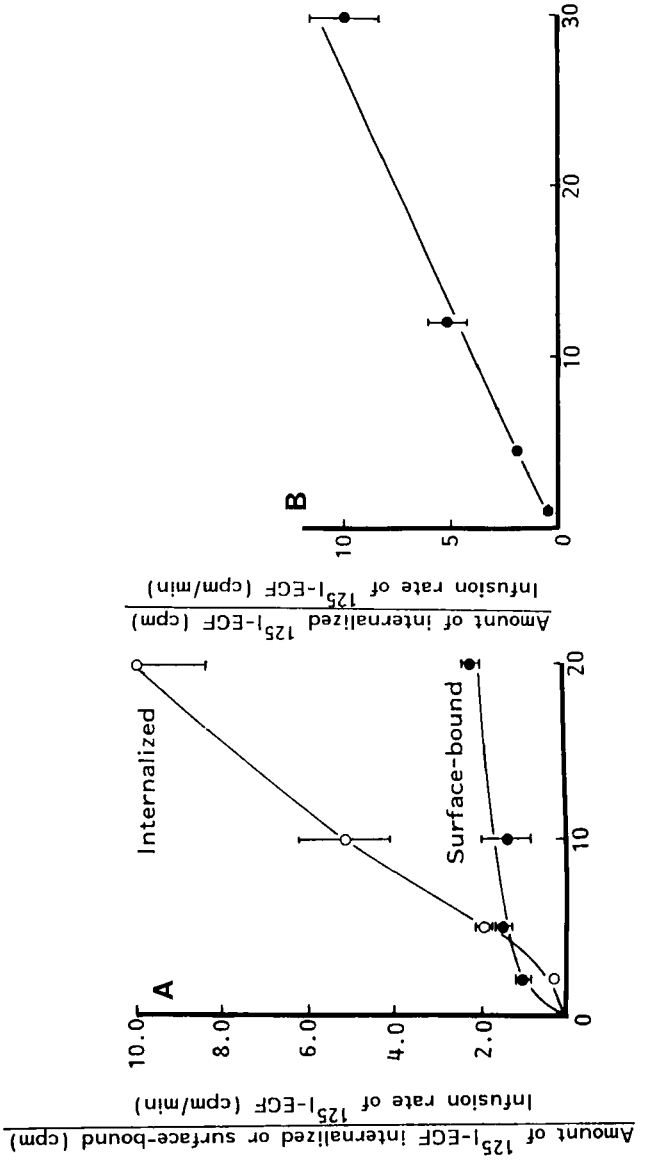

FIGURE 3

(A) Time profiles of acid-washable (●) and acid-resistant (○) radioactivities after the single-pass perfusion of tracer concentration (0.03 nM) ^{125}I-EGF. (B) A plot for the estimation of internalization rate constant (8).

Phenylarsine oxide (PAO) is known as an inhibitor of polypeptide internalization. We determined the k_{int} value by means of a similar technique in the presence or absence of PAO in perfused liver (9), isolated hepatocytes (9), and cultured hepatocytes (10). As a result, PAO decreased the k_{int} value to almost nothing in the presence of only a tracer concentration of ^{125}I-EGF, but not so much in the presence of excess EGF concentration (20 nM) which is enough to occupy almost all cell-surface receptors. The discrepancy of the effects of PAO can be explained if we consider the multiplicity of internalization pathways. That is, some EGF molecules are taken up via relatively non-specific binding sites at excess concentrations of EGF, this non-specific uptake system being relatively PAO-insensitive.

(1-3) Kinetic Analysis of the Receptor Externalization (11,12)

In perfused liver, after most cell-surface receptors were internalized by perfusing an excess (20 nM) concentration of EGF, the perfusate was switched to EGF-free buffer. After that, the recoveries of available cell-surface receptors were chased by the determination of the extraction ratio of ^{125}I-EGF in MID experiments (FIGURE 4) in the presence of cycloheximide to inhibit the insertion of newly-synthesized receptors to the cell-surface (11). As a result, the externalization rate constant (k_{ext}) was estimated to be 0.015 min^{-1}. We also determined the recovery rate of liver cell-surface receptors from their down regulation in vivo (12) and the value was comparable with that in perfused liver.

(1-4) Kinetic Model Representing the RME Process (4,11,13)

Both the kinetic model describing RME and the values of parameters obtained from these experiments are listed in FIGURE 5 and TABLE 1, respectively. Based on this kinetic model, we can quantitate both the hepatic uptake and intracellular transport of ligand and its receptor.

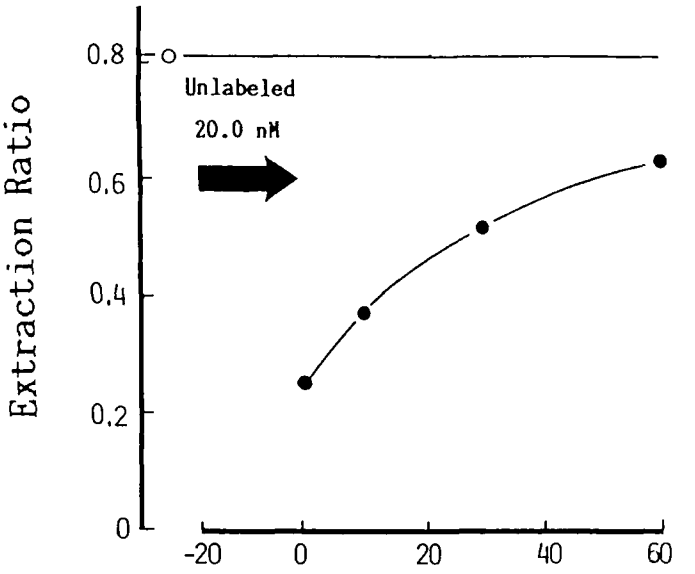

FIGURE 4
Recovery of the hepatic extraction ratio of ^{125}I-EGF 20 min after the perfusion of an excess (20 nM) concentration of unlabeled EGF (11).

(2) Analysis of Hepatic Handling of HGF

(2-1) Mechanism of Hepatic Handling of HGF (13-15)

HGF is the most potent mitogen for mature hepatocytes, with a maximal activity 2 or 3 times higher than that of EGF (16-18). Therefore, it is expected that HGF will be developed as a treatment for certain types of liver diseases.

HGF receptor on primary cultured rat hepatocytes has a relatively high affinity for HGF, with a dissociation constant of 20-30 pM (19) which is approximately 1/100 that of the EGF receptor. On the other hand, the binding capacity is low (500-600 sites/cell, approximately 1/200 that of EGF receptor), and non-specific binding to cell-surface was relatively high (19) even at physiological concentration (<10 pM). This binding is considered to represent the binding to a heparin-like substance on the cell-surface (20) and/or in the extracellular

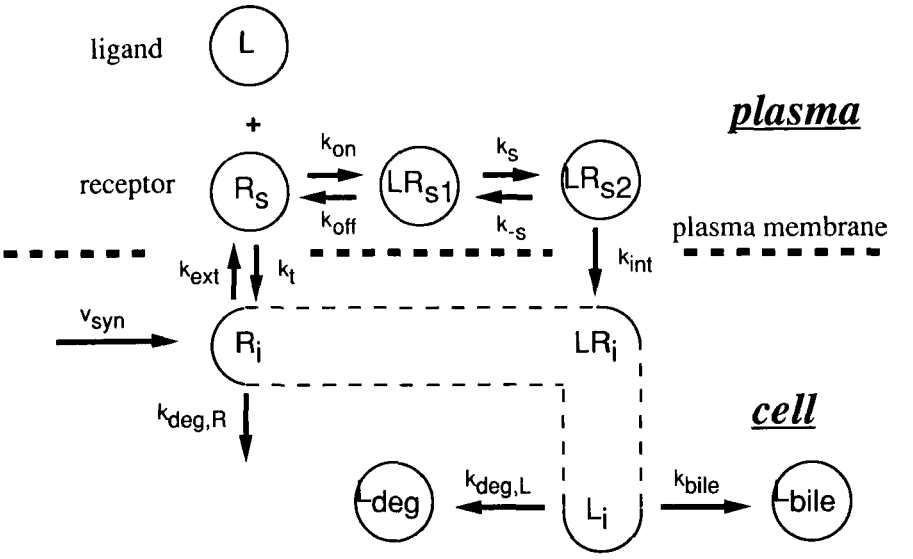

FIGURE 5

Kinetic model describing the receptor-mediated endocytosis of EGF (4,11).

The abbreviations are as follows:

k_{on}: association rate constant of ligand-receptor complex,

k_{off}: dissociation rate constant of ligand-receptor complex,

k_s: sequestration rate constant of ligand-receptor complex from shallow to deep compartment in the membrane,

k_{-s}: transfer rate constant of ligand-receptor complex from deep to shallow compartment in the membrane,

k_t: internalization rate constant of unoccupied receptor,

k_{int}: internalization rate constant of occupied receptor,

k_{ext}: externalization rate constant of intracellular receptor,

$k_{deg,L}$: degradation rate constant of intracellular ligand,

k_{bile}: biliary excretion rate constant of intracellular ligand,

$k_{deg,R}$: degradation rate constant of intracellular receptor,

v_{syn}: de novo synthesis rate of receptors.

(L): ligand,

(R_s): unoccupied cell-surface receptor,

(R_i): unoccupied receptor inside the cell,

(LR_{s1}): ligand-receptor complex in the shallow compartment of plasma membrane,

(LR_{s2}): ligand-receptor complex in the deep compartment of plasma membrane,

(LR_i): ligand-receptor complex inside the cell,

matrix (21), since HGF is basic and has a strong affinity for heparin.

We attempted to separately determine receptor binding and non-specific binding of HGF (13). That is, after the perfusion of tracer ^{125}I-HGF and the following wash with HGF-free buffer, the perfusate was switched to an ice-cold buffer containing heparin, and the outflow was collected (13). As a result, the heparin-washable (HW) radioactivity emerged in the outflow by the heparin-washing (FIGURE 6A), which may represent ^{125}I-HGF bound to a heparin-like substance. After that, an acid buffer (pH 3.0) was perfused and collected. The heparin-resistant and acid-washable (HRAW) radioactivity was released by the following acid-washing (FIGURE 6A). It was suggested that this HRAW radioactivity may mainly represent ^{125}I-HGF bound to its receptor, since the HRAW binding was reduced in the presence of an excess (135 pM) concentration of HGF (FIGURE 6B). The heparin-resistant and acid-resistant radioactivity remained in the liver is considered to represent internalized ^{125}I-HGF (FIGURE 6A). Since the internalized ^{125}I-HGF decreased in the presence of either excess HGF (FIGURE 6B) or PAO (FIGURE 6C), it was suggested that RME contributes to HGF internalization (13). However, some internalization of ^{125}I-HGF was still observed in the presence of either excess HGF (FIGURE 6B) or PAO (FIGURE 6C), suggesting the existence of a low-affinity and PAO-insensitive internalization pathway for HGF (13), as in case of EGF internalization (9,10).

(2-2) Development of DDS for HGF Based on the Mechanism of its Hepatic Handling

The fact that the plasma half-life of HGF is very short (≈ 4 min)(13,14) may become a barrier against its clinical application. Therefore, we attempted to develop a DDS for HGF aimed at decreasing the clearance of HGF, in consideration of the mechanism of the hepatic handling of HGF. To decrease the non-specific binding of HGF to heparin-washable binding sites,

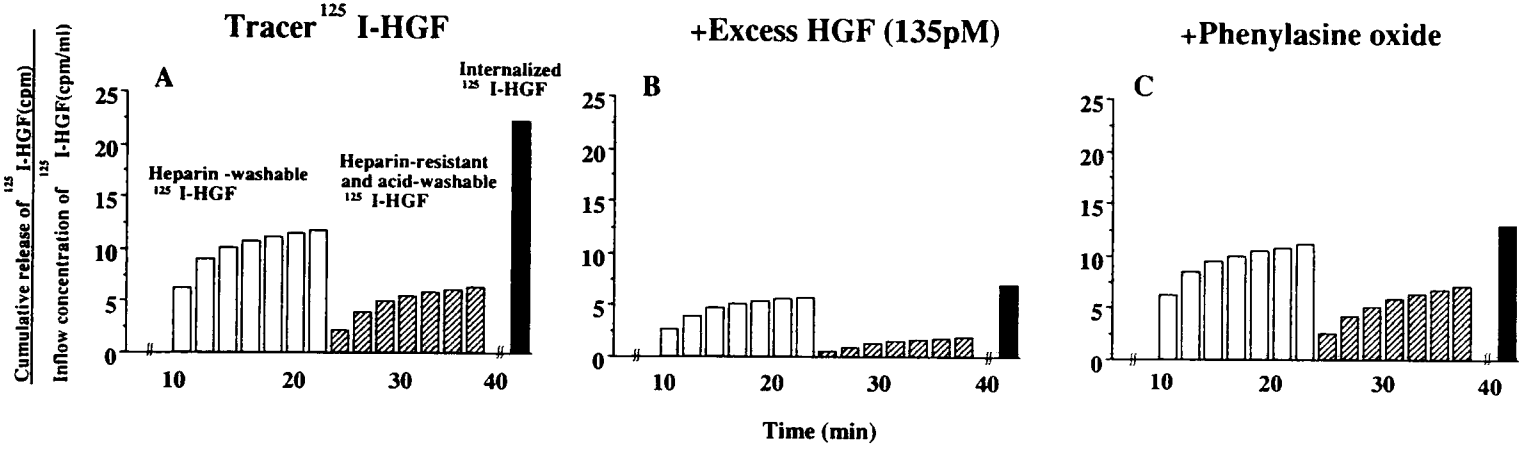

FIGURE 6

Time profiles of heparin-washable, heparin-resistant and acid-washable ^{125}I-HGF recovered in the outflow and internalized ^{125}I-HGF remaining in the liver (14). Tracer ^{125}I-HGF without (A) or with 135 pM unlabeled HGF (B) or phenylarsine oxide (C) was perfused for 15 min to the liver.

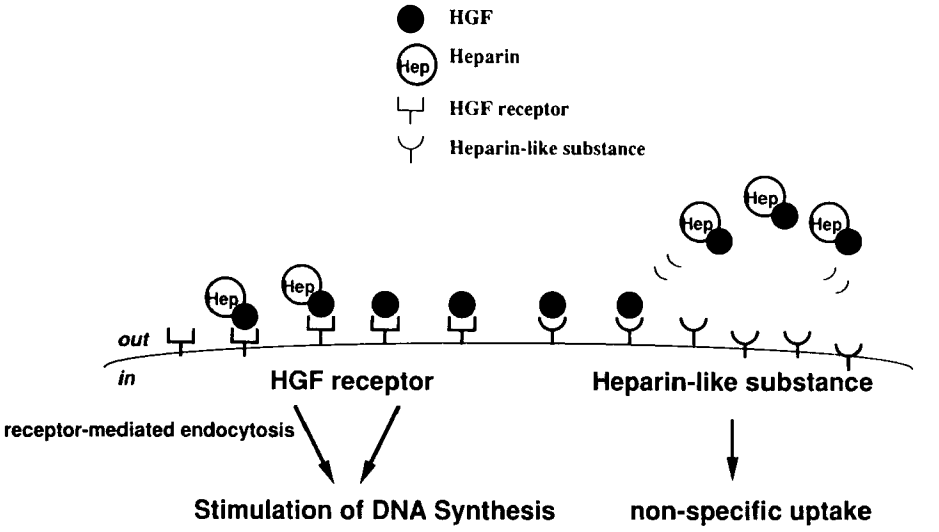

FIGURE 7
Schematic diagram for hepatic handling of HGF alone or heparin-HGF complex.

we administered HGF previously bound to heparin to the body. The area under the plasma concentration-time profiles of trichloroacetic acid-precipitable radioactivity was 2-3 times larger after the administration of such a heparin-^{125}I-HGF complex than that after the administration of ^{125}I-HGF only, suggesting a decrease in HGF clearance. On the other hand, the heparin-HGF complex retained the hepatocyte proliferative activity of HGF, assessed by the induction of DNA synthesis in cultured rat hepatocytes, although its activity was somewhat reduced compared to HGF only. From these results, a simplified model describing the hepatic handling of HGF and the heparin-HGF complex was fashioned (FIGURE 7). HGF molecules carried in the blood flow can bind to both HGF receptor and a heparin-like substance, followed by the internalization via each binding site (FIGURE 7). On the other hand, the binding of the heparin-HGF complex to a heparin-like

substance is reduced by the complex formation (FIGURE 7). The complex, however, still can bind to HGF receptor with relatively lower affinity than HGF alone, followed by the induction of DNA synthesis (FIGURE 7).

(3) Drug Targeting by RME

(3-1) Concept

The features of polypeptide receptor are: (i) relatively high affinity for the ligand, (ii) strict requirements for ligand structure, and (iii) dynamic transport between the cell-surface and intracellular space.

Drug targeting via RME can be accomplished in the following manner: (i) The polypeptide receptor specifically localized in the tissue or the cell to which we want to target drug is found out; (ii) The objective drug is once conjugated to a vector which can bind to the receptor, and is then administered to the body; (iii) The drug-vector complex is taken up via RME by the target cell. The polypeptide itself, its analog, or an anti-receptor antibody can be used as the vector for the receptor. The vector is often conjugated to the objective drug either covalently or noncovalently.

(3-2) Examples of the Drug Targeting

Elevated levels of EGF receptors have been demonstrated in many types of tumor cell such as the squamous carcinoma cell in esophageal and lung tumors (22). Therefore, immunotoxin -- a complex of monoclonal antibodies against the EGF receptor and cytotoxic molecules such as recin or gelonin -- may show tumor-specific toxicity. Such an immunotoxin exhibited more anti-cancer activity than cytotoxic molecules alone both in the in vitro cultured cell system (23) and in in vivo tumor-transplanted nude mice (24). The transferrin receptor is also known to be over-expressed in tumors compared to normal adjacent tissues, the tumor-specificity of immunotoxin made with anti-transferrin receptor having also been examined

(25). Immunotoxin has reportedly been applied in clinical use (26), but demonstrated serious side effects, since it was made with mouse antibody and caused the production of human antibody against mouse antibody in the circulating blood. A technique for the mass production of human antibody is expected to be developed.

Drug targeting using monoclonal antibody to normal tissue was also attempted. In general, it is difficult to deliver hydrophilic and/or large molecules into the brain, since the blood-brain barrier restricts the movement of molecules between the circulating blood and the brain interstitial space. However, some polypeptide receptors such those for insulin and transferrin are localized on the surface of brain capillary endothelial cells. Therefore, it may be possible to deliver some drug into the brain via such receptors. The monoclonal antibody, designated OX-26, against the transferrin receptor is one of the vectors which distributes selectively into the brain (27).

Receptor-mediated gene delivery to liver parenchymal cells was attempted by Wu et al (28-30). These days, the focus is on gene therapy for the treatment of inherited diseases, which are caused by a deficiency in some gene or gene expression. However, DNA oligonucleotide is a hydrophilic and large molecule, and its penetration through the plasma membrane is restricted. For the expression of exogenously administered DNA, it has to be delivered into the cells using a certain DDS. The Wu group selected a conjugate of asialoglycoprotein whose receptors are specifically localized on liver parenchymal hepatocytes, and polylysine which is a cationic compound and can bind to oligonucleotide electrostatically, as the vector for gene delivery (FIGURE 8)(28-30). Incubation of this protein-polylysine complex with target DNA oligonucleotide led to the formation of the DNA-protein complex. This technique can minimize the decrease in DNA activity and make it possible for DNA to dissociate easily from the vector in the intracellular

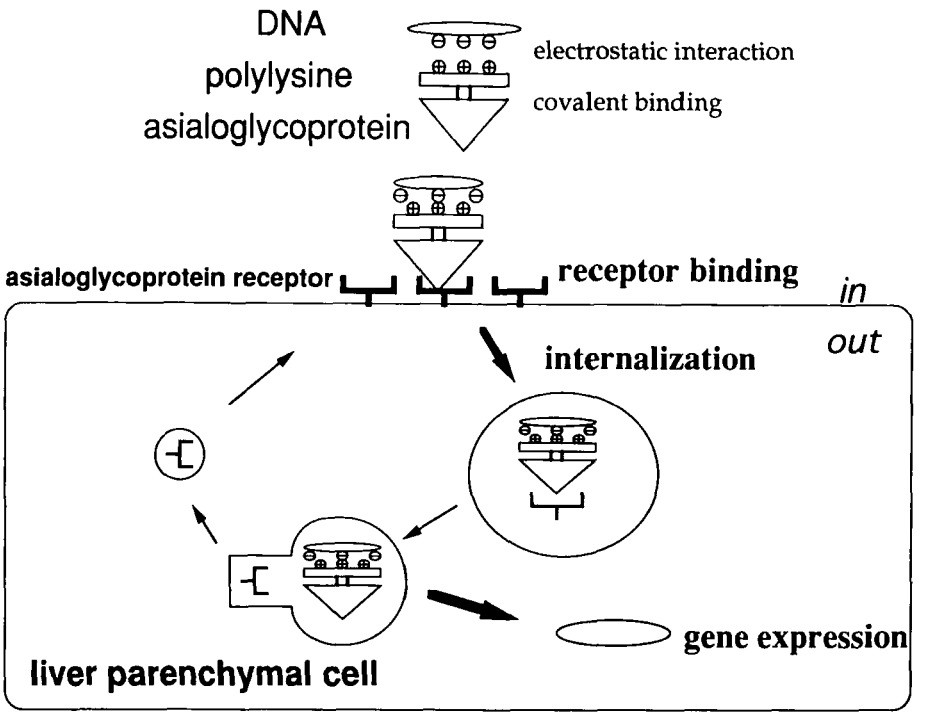

FIGURE 8
Schematic diagram for the strategy of the targeting of oligonucleotide to hepatocyte by asialoglycoprotein receptor-mediated endocytosis (28-30).

vesicles, since the binding between oligonucleotide and protein is not covalent (FIGURE 8). Using this technique, albumin mRNA was expressed in liver, and albumin was detected in the circulating blood in the Nagase analbuminemic rat in vivo after the systemic administration of plasmid containing the gene for human serum albumin (28). In addition, the treatment of DNA-protein complex containing the low density lipoprotein (LDL) receptor gene led to transient improvement of hypercholesterolemia in LDL receptor-deficient rabbits (29). These results suggested that this technique will make gene delivery into the liver possible.

(3-3) Consideration of the Efficiency of Drug Targeting Based on the Kinetic Model for RME

Based on the kinetic model describing RME (FIGURE 5), we will discuss the preferable combination of ligand and receptor in view of the efficiency of drug targeting.

In this analysis we considered only the steady state condition, and made the following assumptions:

(i) Only one compartment exists for the ligand-receptor complex on the cell-surface.

(ii) The values of some kinetic parameters (k_{ext}, $k_{deg,R}$, V_{syn}) in the presence of ligand are equal to those in its absence. In other words, ligand does not either induce or reduce each process in RME except the internalization process.

(iii) The extraction of the ligand in the target organ is very low. That is, ligand concentration in the capillary space is almost equal to the plasma concentration (C_p) and is constant in the steady state.

Since receptor density both on the cell-surface and in the cell can be considered to be at steady-state in the absence of ligand, the following equations hold:

$$dR_s/dt = -k_t R_s + k_{ext} R_i = 0 \quad \text{Eq. (3)}$$

$$dR_i/dt = k_t R_s + V_{syn} - (k_{deg,R} + k_{ext}) R_i = 0 \quad \text{Eq. (4)}$$

Therefore,

$$R_s(0) = k_{ext} V_{syn} / (k_t k_{deg,R}) \quad \text{Eq. (5)}$$

$$R_i(0) = V_{syn} / k_{deg,R} \quad \text{Eq. (6)}$$

where $R_s(0)$ and $R_i(0)$ are cell-surface and intracellular receptor density in the absence of ligand, respectively. The definitions of the other parameters are listed in the legend of FIGURE 5.

In the presence of ligand, the following mass balance equations hold in the steady state condition:

$$d(LR_s)/dt = k_{on} (R_s - (LR_s)) C_p - (k_{off} + k_{int})(LR_s) = 0 \quad \text{Eq. (7)}$$

$$dR_s/dt = -k_t (R_s - (LR_s)) + k_{ext} R_i - k_{int} (LR_s) = 0 \quad \text{Eq. (8)}$$

$$dR_i/dt = k_{int} (LR_s) - (k_{deg,R} + k_{ext}) R_i + k_t (R_s - (LR_s)) + V_{syn} = 0 \quad \text{Eq. (9)}$$

The uptake (internalization) rate of the ligand can be expressed by:

$$V_{uptake} = k_{int} (LR_s)$$
$$= k_t R_s (0) C_p / [(k_t / k_{int}) \times \{(k_{off} + k_{int}) / k_{on}\} + C_p]$$
$$= V_{max,app} C_p / (K_{m,app} + C_p) \quad \text{Eq. (10)}$$

where

$$K_{m,app} = (k_t / k_{int}) \times \{(k_{off} + k_{int}) / k_{on}\} \quad \text{Eq. (11)}$$

$$V_{max,app} = k_t R_s (0)$$
$$= k_{ext} V_{syn} / k_{deg,R} \quad \text{Eq. (12)}$$

Based on Eq. (10), the uptake rate of the ligand cannot exceed $V_{max,app}$ although the uptake rate increases as ligand concentration increases. This equation indicates that a proportional increase in drug in the target cell cannot be expected when the plasma concentration of the ligand is set up to higher level than $K_{m,app}$ value. In addition, Eq. (10) means that the preferable combination of ligand and receptor has a small value of $K_{m,app}$ and a large value of $V_{max,app}$. The $K_{m,app}$ decreases as either k_{on} increases or k_{off} decreases, that is, when the dissociation constant ($Kd = k_{off} / k_{on}$) between ligand and receptor is small.

The $K_{m,app}$ can be expressed as:

$$K_{m,app} = K_m / \alpha \quad \text{Eq. (13)}$$

$$\alpha = k_{int} / k_t \quad \text{Eq. (14)}$$

$$K_m = (k_{off} + k_{int}) / k_{on} \quad \text{Eq. (15)}$$

where α is a constant representing the acceleration of the internalization rate constant of the receptor induced by the binding to the ligand. The efficiency of drug targeting increases as the α value increases. That is, when the ligand sharply induces receptor internalization, the efficiency is high.

The $V_{max,app}$ value is proportional to the $R_s(0)$ value. This means that the efficiency of drug targeting is high when we target densely expressed receptors on the cell-surface. Both the receptor externalization rate constant (k_{ext}) and the constitutive internalization rate constant (k_t) are positively correlated to the V_{uptake} value. This indicates that the receptor

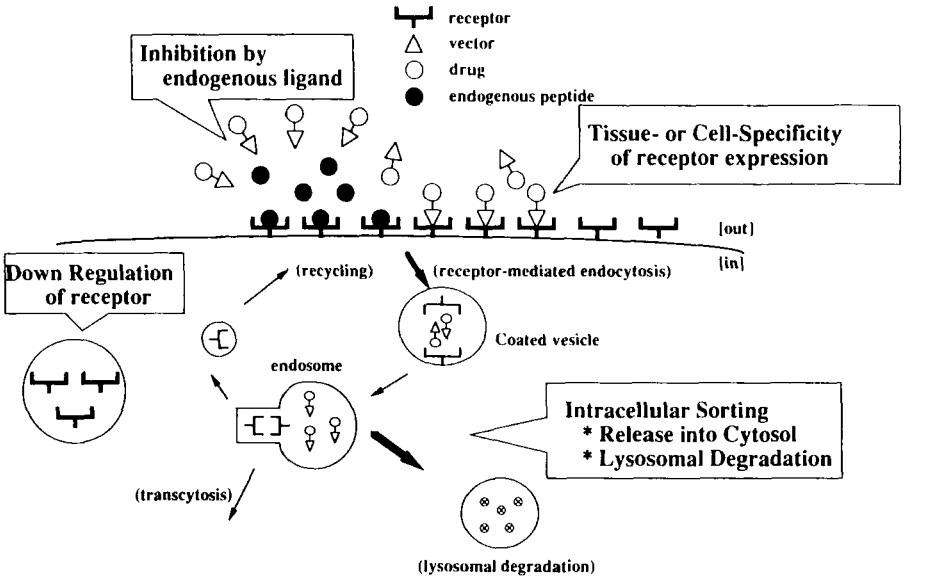

FIGURE 9
Critical aspects of drug targeting by RME.

which is transported rapidly between the cell-surface and cell-interior can be used as a preferential targeting site.

(3-4) Critical Aspects of the Drug Targeting by RME

The drug delivery by RME is one of the valid strategy for the drug targeting to the organ or the cells which highly express receptors. However, there are some critical points which have to be resolved (FIGURE 9).

One of the most critical points is the specificity of the localization of receptors. Although a certain type of receptor is expressed densely in tumor cell, it exists also in normal tissue. Therefore, drug targeting by RME cannot have complete cell- or tissue-specificity. For example, EGF receptors are densely expressed also in epithelial cells both in liver and kidney. It is possible that conjugation of a drug to a vector which binds a receptor leads not only to an increase in the efficiency of drug

targeting, but also to an increase in the uptake of a drug by liver or kidney.

The other critical point is the inhibition of receptor binding by endogenous ligand. In the diseased condition, it may be possible that endogenous polypeptide level in plasma increases, resulting in the occupation of receptors. In such a condition, it is difficult to target a drug with RME, except when we use a monoclonal antibody which would recognize different sites of a receptor molecule from those recognized by endogenous ligand.

There are some critical aspects related to the dynamic transport of receptor itself. One is the down regulation of receptors. For example, asialoglycoprotein receptors which can be used for targeting to the liver, are down-regulated in hepatic injury (31). Therefore, it may be difficult to use the receptor as a target in such a diseased condition.

When we target a drug with RME, the drug is taken up by the cell within a vesicle. Therefore, if the drug is active only in cytosol, it must once pass through the membrane of the vesicle before it reaches the cytosol to exert its pharmacological effect. It is expected that a method which can release a drug in the vesicle into the cytosol will be developed.

The other critical aspect originates from the intracellular sorting of the ligand. At least a portion of the ligand once internalized is transported into lysosome, with resultant degradation. Therefore, it is difficult to target a drug which is easily degraded or inactivated in lysosomes. However, certain types of polypeptide receptors are known to be recycled back to cell-surface and/or transcytosed to the opposite side. This fact hints to us the existence of some sorting mechanism which can prevent internalized molecules from lysosomal degradation. If we can control such a sorting mechanism, the efficiency of the targeting by RME can be greatly advanced.

Although there are some critical aspects yet to be completely solved, we expect to see a rapid development of

specific and efficient drug targeting by RME after their resolution.

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