DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY, 20(4), 581-590 (1994)

TARGETING DELIVERY OF PROTEIN DRUGS BY CHEMICAL MODIFICATION

Mitsuru Hashida, Makiya Nishikawa, Fumiyoshi Yamashita,
and Yoshinobu Takakura
Faculty of Pharmaceutical Sciences, Kyoto University
Sakyo-ku, Kyoto 606-01, Japan

ABSTRACT

In vivo disposition profiles of protein derivatives having various chemical modifications were systematically compared in mice based on the clearance concept. Proteins such as bovine γ-globulin (IgG), bovine serum albumin (BSA), superoxide dismutase (SOD), soybean trypsin inhibitor (STI), and chicken egg white lysozyme (LZM) were 1)conjugated with polyethylene glycol (PEG) and dextran to increase molecular size, 2) conjugated with carboxymethyl-dextran (CMD) and diethylaminoethyl-dextran (DEAED) or coupled with diaminohexane or succinic acid to introduce electric charges, and 3) modified with galactose (Gal) and mannose (Man) moieties to bestow an affinity for receptor-mediated endocytosis in cells. By applying these modifications, in vivo disposition features of proteins were extensively changed; i.e., in the case of SOD, conjugation with CMD and PEG prolonged its circulation half-life more than 100 times but cationized SOD showed remarkable accumulation on the surface of the liver tissue. In addition, specific targeting to the parenchymal cells of the liver was demonstrated in Gal-SOD, while, Man-SOD and succinylated SOD showed rapid uptake by the nonparenchymal cells. These results revealed the utility of chemical modification for controlling in vivo disposition of proteins.

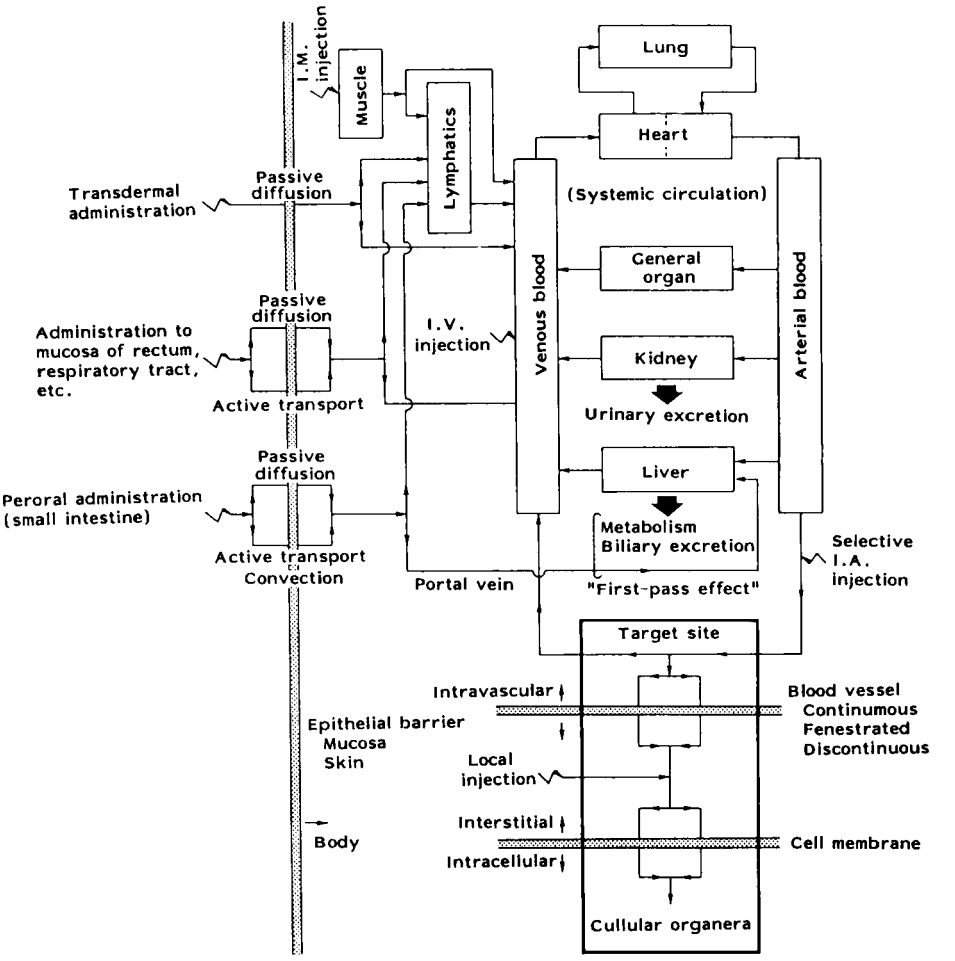

FIGURE 1

A scheme illustrating movement pathways and mechanism of each transport process of drugs in the body

INTRODUCTION

Drug delivery system is simply defined as a technology to precisely control in vivo fate of drugs, which can be summarized as a semi-closed system shown in figure 1, for achieving optimal therapeutic efficacy. In order to realize this goal, we should elucidate the molecular mechanism of each transport process and understand total characteristics of the system. Based on these considerations, we have systematically investigated in vivo disposition of macromolecules (1, 2).

On the other hand, the clinical application of biologically active proteins is sometimes hampered due to substantial problems relating to their biopharmaceutical (absorption, distribution, metabolism, etc.) properties as well as pharmaceutical (stability, solubility, aggregability, etc.) and immunological (antigenicity, immunogenicity, etc.) characteristics. One of the promising approaches to solve these problems may be chemical modification with various molecular species and through these approaches, not only an improvement in general pharmacokinetic features of proteins but also a specific delivery to the target site can be achieved.

In our series of investigation, systemic disposition profiles of macromolecules were explored in relation to their physicochemical and biological properties, and a strategy for designing targeting system was constructed based on the obtained findings (3, 4). In this paper, the effectiveness of this approach will be discussed through investigations of in vivo pharmacokinetic characteristics of various derivatives of model proteins.

MATERIALS AND METHODS

Model Proteins

Bovine γ-globulin (IgG: MW=150 kDa), bovine serum albumin (BSA: MW=67 kDa), soybean trypsin inhibitor (STI: MW=20 kDa), and chicken egg white lysozyme (LZM: 14 kDa) were obtained from Sigma, St Louis, U.S.A. Recombinant human superoxide dismutase (111-Ser: SOD: MW=32 kDa) was kindly supplied by Asahi Chemicals Co., Tokyo, Japan. Conjugation of proteins with dextran derivatives was carried out by a periodate oxidation method (5). Activated polyethylene glycol (PEG: MW=10 kDa) obtained from Seikagaku Kogyo, Co., Tokyo, Japan was reacted with proteins as reported previously (5). Cationic diethylaminoethyl-dextran (DEAED: MW=70 kDa) and anionic carboxymethyl-dextran (CMD: MW=70 kDa) were synthesized as reported previously. Cationized and anionized proteins were also synthesized by coupling with diaminohexane and succinic acid as reported previously (6). Coupling of galactose (Gal) and mannose (Man) was carried out according to the method of Lee et al. (7). Proteins were radiolabeled with ^{111}In using a bifunctional chelating agent, diethylenetriaminepentaacetic acid (DTPA) anhydride, according to the method of Hnatowich et al. (8) for in vivo study.

In Vivo Distribution Experiment

Male ddY mice (25–28 g) were obtained from Shizuoka Agricultural Co-operative Association for Laboratory Animals, Shizuoka, Japan. Mice received various doses of radiolabeled protein derivatives in saline by tail vein injection. At adequate time periods after injection, blood was collected from the vena cava under ether anesthesia and mice were sacrificed. The heart, lung, liver, spleen, kidney, and muscle were excised, rinsed with saline, weighed, and subjected to radioactivity counting with a well-type NaI-scintillation counter (ARC-500, Aloka Co, Tokyo, Japan). Contamination of plasma in tissue samples was corrected using distribution data of ^{111}In-labeled BSA at ten min after intravenous injection.

Calculation of Organ Uptake Clearances

The tissue distribution was evaluated using an organ uptake clearance (CLorg) described previously (3). In the early stage after injection, the efflux of the ^{111}In radioactivity from the organ is considered to be negligible and CLorg can be calculated by dividing the amount of radioactivity in the organ at an appropriate interval of time by the area under the plasma concentration–time curves (AUC) up to the same time point.

RESULTS AND DISCUSSION

In Vivo Disposition Characteristics of Various Model Macromolecules

In order to accomplish effective targeting, the protein drugs should be rationally designed not only to have high affinity to the target site but also to escape from undesirable removal such as the uptake by the reticuloendothelial system (RES). Thus an understanding of the factors which regulate in vivo disposition of macromolecules is an important aspect. On the basis of these considerations, we have evaluated pharmacokinetic characteristics of various model macromolecules based on a clearance concept. Figure 2 summarizes the latest information about relationship between physicochemical properties of macromolecules and their hepatic uptake and urinary excretion susceptibility in mice which are essential determinant for whole body level disposition. Concerning the effect of molecular size, small macromolecules with molecular weights of approximately 10 kDa are shown to have large excretion clearances (CLurine) which are close to the glomerular filtration rate. On the other hand, macromolecules with molecular weights of larger than 70 kDa show CLurine

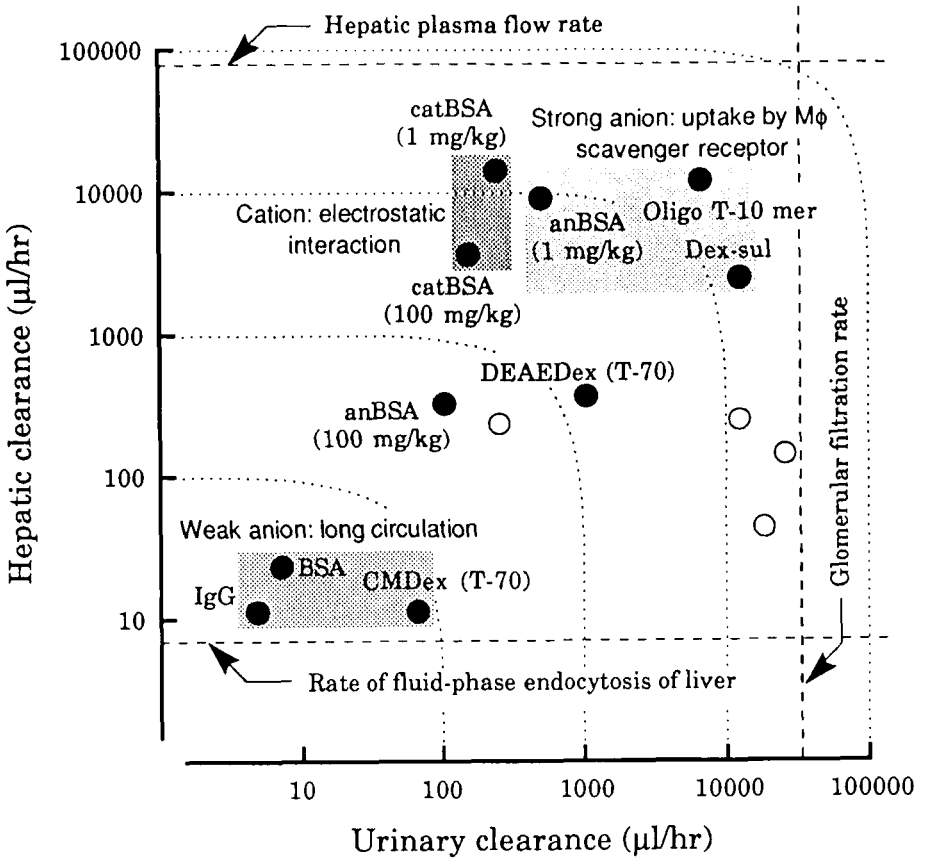

FIGURE 2

Hepatic and urinary clearances of model macromolecules in mice

values which are less than one hundred times smaller than those of small molecules. Thus, molecular size is suggested to be the major determinant of urinary excretion although the glomerular endothelium also works as a charged barrier.

Figure 2 is focused on the effect of electric charges of macromolecules. Molecules with molecular weights of more than 70 kDa and weak anionic charge show very small CLliver values as well as CLurine. On the contrary, cationized macromolecules show large CLliver values due to electrostatic interaction with the surface of the liver cells (6). In addition, strongly anionized macromolecules also demonstrated large hepatic uptake by the scavenger–receptor of macrophages

and endothelial cells. From these results, it can be concluded that macromolecules with adequate molecular size and weak anionic nature will show prolonged retention in the plasma circulation and then large AUC.

We have also demonstrated that BSA can be successfully delivered to the liver parenchymal and non-parenchymal cells by the direct attachment of galactose and mannose moieties, respectively (9). At lower doses (0.1 mg/kg), CLliver values of Gal-BSA and Man-BSA achieved at about 83.4 and 19.3 (ml/hr), respectively. Here, the former value is almost similar to the hepatic plasma flow rate.

Targeting of SOD with Chemical Modification

So far, we have shown that in vivo disposition profiles of macromolecules are extensively changed depending on their physicochemical and biological properties. Among the various approaches to solving the problems inherent to protein drugs such as a short plasma half-life, therefore, chemical modification utilizing moieties with various characteristics is the most promising. In particular, modification of proteins with carbohydrates seems to be attractive due to their ability to add various transport properties to proteins and because of their high biocompatibilities. Based on these findings, we have synthesized five types of SOD derivatives such as PEG-SOD, SOD-CMD, SOD-DEAED, Gal-SOD, and Man-SOD and evaluated their disposition characteristics in mice (10).

Figure 3 summarizes hepatic uptake and urinary excretion characteristics of these derivatives. SOD itself is characterized by a large CLurine since it is a relatively small protein having a molecular weight of 32 kDa which is susceptible to glomerular filtration. The CLliver value was markedly increased by conjugating with DEAED, but urinary excretion was also reduced in this case. In contrast, conjugation of CMD to SOD reduced both hepatic uptake and urinary excretion and thus resulted in prolonged plasma retention of enzymatic activity. Similar results were obtained for PEG-SOD, as reported by several groups (11). On the other hand, by introducing monosaccharides to SOD, the value of CLliver was 100 to 1000 times increased without significant change in CLurine. Their CLliver values at a dose of 0.1 mg/kg were nearly equal to the hepatic plasma flow rate, suggesting almost complete uptake of Gal-SOD and Man-SOD in the liver.

Based on these findings, the SOD derivatives developed in this study can be classified as follows: 1) long-circulating type (SOD-CMD, PEG-SOD); 2) cell-surface targeting type (SOD-DEAED); 3) intracellular targeting type for

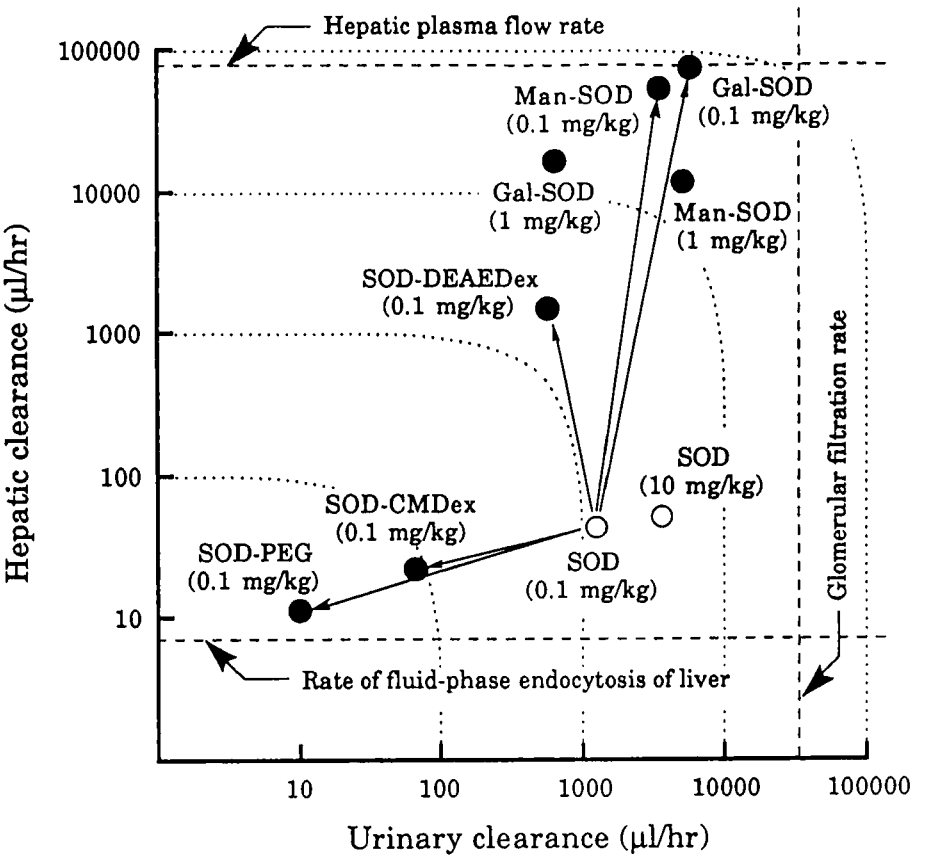

FIGURE 3

Hepatic and urinary clearances of SOD derivatives in mice

hepatocyte (Gal–SOD) and resident macrophages and endothelial cells in the liver and spleen (Man–SOD). In a pharmacological test, Man–SOD showed a promising therapeutic effect on a rat hepatic ischemia–reperfusion injury (12). These SOD derivatives also demonstrated unique disposition profiles in the rat isolated perfused kidney (13).

Molecular Design of Protein Derivatives for Their Targeting Utilizing Carbohydrate Recognition Mechanism

In preceding discussion, a potential of chemical modification especially of the introduction of sugar moieties for protein targeting has been demonstrated. In

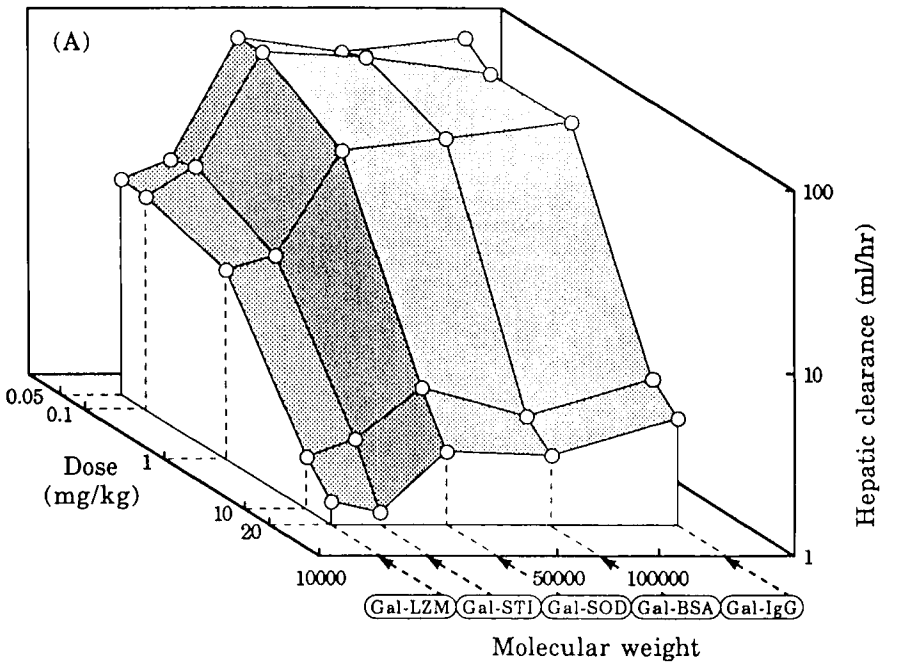

FIGURE 4

Relationship between molecular weights of original proteins and hepatic uptake behaviors of their galactosylated derivatives at different doses in mice

order to actualize optimal targeting, however, more precise molecular design of proteins would be required, since, for example, biodistribution of glycosylated albumin was reported to differ extensively with molecular properties such as the extent of sugar substitution (14). In a series of investigations on the effects of molecular structures of glycosylated proteins on their in vivo disposition, we have elucidated the effect of molecular sizes of original proteins on CLliver and CL_{urine} values of their derivatives. In this study, five proteins with different molecular weights were galactosylated at relatively similar galactose contents (4.4 – 7.7 % in weight). Figure 4 summarizes CLliver values of five galactosylated proteins at five different doses. As reported previously (9), hepatic uptake of all compounds followed non-linear kinetics, suggesting the participation of receptor-mediated endocytosis. Among five compounds, Gal-IgG, Gal-BSA, and Gal-SOD gave large CLliver values at doses lower than 1 mg/kg. On the contrary, Gal-STI and Gal-LZM showed relatively small CLliver values even at low doses. In addition,

since these small protein derivatives have large CLurine values, the final achievement of hepatic targeting was limited to about 30 % of dose, although larger derivatives afforded about 70 - 80 % of dose. The absolute numbers of galactose residues in Gal-STI and Gal-LZM are 6 and 4.8, respectively, while the other three compounds have those higher than 14. Therefore, it seems that there is a kind of threshold in total numbers or density of galactose residues in one molecules. Thus, more detailed and systematic information is required for rational design of protein derivatives.

CONCLUSION

The present discussion reveals the utility of chemical modification, especially sugar moiety introduction, for controlling the in vivo disposition of proteins. Through these approaches, pharmacokinetic information at a whole body level obtained based on the clearance concept can make significant contribution in the rational design of a targeting system.

REFERENCES

1. H. Sezaki and M. Hashida, CRC Crit. Rev. Ther. Drug Carrier Syst., 1, 1 (1984).
2. H. Sezaki, Y. Takakura, and M. Hashida, Adv. Drug Delivery Rev., 3, 247 (1989).
3. Y. Takakura, T. Fujita, M. Hashida, H. Sezaki, Pharm. Res., 7,339 (1990)
4. M. Hashida, Y. Takakura, and H. Sezaki, in "Perspectives in Medicinal Chemistry," B. Testa, E. Kyburz, W. Fuhrer, and R. Giger, eds., Verlag Helvetica Chimica Acta, Basel, 1993, p. 545.
5. T. Fujita, Y. Yasuda, Y. Takakura, M. Hashida, and H. Sezaki, J. Controlled Release, 11, 149 (1990).
6. K. Nishida, K. Mihara, T. Takino, S. Nakane, Y. Takakura, M. Hashida, and H. Sezaki, Pharm. Res., 8, 437 (1991).
7. Y.C. Lee, C.P. Stowell, and M.J. Krantz, Biochemistry, 15, 3956 (1976).
8. D. Hnatowich, W.W. Layne, and R.L. Childs, Int. J. Appl. Radiat. Isot., 12, 327 (1982).
9. M. Nishikawa, Y. Ohtsubo, J. Ohno, T. Fujita, Y. Koyama, F. Yamashita, M. Hashida, H. Sezaki, Int. J. Pharmaceut., 85, 75 (1992).

10. T. Fujita, M. Nishikawa, C. Tamaki, Y. Takakura, M. Hashida, and H. Sezaki, J. Pharmacol. Exp. Ther., 263, 971 (1992).
11. F. Fuertges and A. Abuchowski, J. Controlled Release, 11, 139 (1990).
12. T. Fujita, H. Furitsu, M. Nishikawa, Y. Takakura, H. Sezaki, and M. Hashida, Biochem. Biophys. Res. Commun., 189, 191 (1992).
13. Y. Takakura, K. Mihara, and M. Hashida, J. Controlled Release, in press.
14. D.R. Vera, K.A. Krohn, R.C. Stadalnik, P.O. Sheibe, J. Nucl. Med., 25, 779 (1984).