Development and Pharmacokinetics of Galactosylated Poly-L-Glutamic Acid as a Biodegradable Carrier for Liver-Specific Drug Delivery

Hideki Hirabayashi,[1] Makiya Nishikawa,[1] Yoshinobu Takakura,[1] and Mitsuru Hashida[1,2]

Received December 30, 1995; accepted February 27, 1996

Purpose. A biodegradable carrier for the liver-specific delivery of drugs was developed using poly-L-glutamic acid (PLGA) modified with galactose (galactosylated PLGA or Gal-PLGA), and its feasibility was investigated in mice.

Methods. ^{111}In-PLGA and ^{111}In-Gal-PLGAs were injected in mice and their distribution and biodegradation properties were studied.

Results. After intravenous injection, ^{111}In-PLGA was rapidly eliminated from the plasma and recovered mainly in the kidneys and urine. Approximately 15% of the dose was recovered in the liver, predominantly in the nonparenchymal cells. ^{111}In-Gal-PLGAs were taken up by the liver parenchymal cells. Derivatives having 16 or more galactose residues were taken up by the liver to a higher extent (>60% of the dose). The hepatic clearance of ^{111}In-Gal-PLGAs correlated with their number of galactose residues. ^{111}In-Gal_{18}-PLGA was degraded into low-molecular weight products in the liver.

Conclusions. The advantageous *in vivo* properties of Gal-PLGA as a liver-specific biodegradable carrier of drugs were demonstrated in mice.

KEY WORDS: drug carrier; hepatic targeting; poly-L-glutamic acid; galactosylation; pharmacokinetics.

INTRODUCTION

Conjugation of drugs to macromolecules is a promising approach to enhance the potency of drugs, by controlling their disposition properties after administration (1). Selective delivery of drugs to target sites can be achieved by reducing nonspecific interaction with normal tissues and by increasing the affinity with the target sites. We previously demonstrated that the *in vivo* fate of macromolecules and drug-macromolecule conjugates can be controlled mainly by their molecular weight and electric charge (2,3). After intravenous administration, weakly anionized and higher molecular-weight macromolecules remain in the blood circulation for a long time (3); this property may be useful for selective delivery of drugs to target tissues after the introduction of proper homing devices to the target.

Carbohydrate receptor-mediated targeting of drugs has been proposed as a potential method for site-specific delivery of drugs, proteins and genes to cells possessing carbohydrate receptors on the cell surface. We have examined the factors affecting the hepatic uptake of glycosylated macromolecules through pharmacokinetic analysis of their *in vivo* disposition after intravenous injection in mice (4,5). It was successful to deliver cytosine arabinoside using galactosylated carboxymethyl-dextran to liver parenchymal cells (6), as well as recombinant human superoxide dismutase (SOD) by direct galactosylation and mannosylation to liver parenchymal and nonparenchymal cells, respectively (7). In addition, hepatic targeting of SOD by galactose or mannose modification enhanced its therapeutic efficacy against hepatic injury induced by ischemia/reperfusion (8).

After delivering drugs to target sites, carriers should be degraded and eliminated from the body with little adverse effects. This property would also lead to the release of drugs from drug-macromolecule conjugates in the target cells. Poly-L-glutamic acid (PLGA) possesses some advantages as a drug carrier such as biodegradability, high water solubility, the presence of multiple carboxyl groups that are easily modified chemically, low immunogenicity and low toxicity. The electric charge of PLGA is also considered to be favorable for controlling its *in vivo* disposition characteristics (3). Although PLGA was used to alter the disposition characteristics and increase the *in vivo* effects of antitumor agents (9,10), there has been little information on designing PLGA derivatives as targetable carriers with suitable *in vivo* disposition characteristics.

In this study, therefore, we synthesized several types of galactosylated PLGA (Gal-PLGA) as candidates for a liver-specific carrier and examined their potential for drug targeting by analyzing the *in vivo* disposition characteristics and the degradation properties in the liver.

MATERIALS AND METHODS

Chemicals

PLGA with an average molecular weight of approximately 25 kDa was purchased from Sigma Chemical Co., St. Louis, Mo, USA. D-galactose and ethylenediamine were obtained from Wako Pure Chemical, Osaka, Japan. Diethylenetriamine-N,N,N′,N″,N″-pentaacetic dianhydride (DTPA anhydride) was obtained from Dojindo Laboratory, Kumamoto, Japan. ^{111}Indium chloride was supplied from Nihon Medi-Physics Co., Takarazuka, Japan. All other chemicals were reagent grade products obtained commercially.

Animals

Male ddY mice (25–28 g) were obtained from the Shizuoka Agricultural Co-operative Association for Laboratory Animals, Shizuoka, Japan.

Synthesis and Characterization of PLGA Derivatives

Attachment of galactose to PLGA was carried out as described previously (5). Briefly, cyanomethyl 1-thiogalactoside was treated with 0.01 M sodium methoxide at room temperature for 24 hr. The solvent was evaporated in vacuo and ethylenediamine was added to the resultant syrup of 2-imino-2-methoxyethyl 1-thiogalactoside (IME-thiogalactoside). After 24 hr at room temperature, PLGA was added into the mixture and the pH of the reaction mixture was adjusted to about 5.0 by addition of 0.1N HCl. Then, 1-ethyl 3-(3-dimethylaminopropyl)carbodiimide was slowly added to the mixture and the pH of the solution was maintained at about 5.0. The mixture was

[1] Department of Drug Delivery Research, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan.
[2] To whom correspondence should be addressed.

0724-8741/96/0600-0880$09.50/0

transferred to dialysis tubing and dialyzed exhaustively against water for 48 hr. The degree of galactosylation was controlled by the molar ratio of the starting reagents, PLGA and IME-thiogalactoside.

The apparent molecular weight of PLGA derivatives was estimated by HPLC gel-filtration chromatography using a Shim-pack Diol-300 column (Shimadzu, Kyoto, Japan). The number of galactose residues and the galactose content in the derivatives were determined by the anthrone-sulfuric acid method.

PLGA derivatives were radiolabeled with ^{111}In using DTPA anhydride as described previously (5). Each radiolabeled derivative was purified by gel-filtration chromatography using a Sephadex G-25 column (1 × 40 cm) and determined to have a specific activity of approx. 37 MBq/mg.

Biodistribution Experiment

Mice received a 1 mg/kg dose of ^{111}In-labeled PLGA or Gal-PLGAs in saline by tail vein injection and were housed in metabolic cages for urine collection. At appropriate intervals after injection, blood was collected from the vena cava under ether anesthesia and plasma was obtained by centrifugation. The heart, lungs, liver, spleen, kidneys and muscle were excised, rinsed with saline, weighed, and examined for radioactivity. The amount of ^{111}In radioactivity in urine was determined by collecting urine both excreted and remaining in the bladder. ^{111}In radioactivity was counted by a well-type NaI scintillation counter (ARC-500, Aloka, Tokyo). Radioactivity originating from the plasma in each tissue sample was corrected using the distribution data of ^{111}In-BSA at 10 min after intravenous injection (4), assuming that BSA was not taken up by tissues during the 10 min period.

Amounts recovered in liver parenchymal (PC) and nonparenchymal cells (NPC) of ^{111}In-labeled PLGA and Gal-PLGA were determined by counting radioactivity in PC and NPC after fractionation by the collagenase perfusion method (11). In addition, ^{111}In-PLGA was co-injected at a 1 mg/kg dose with several BSA derivatives at a dose of 20 mg/kg. At 1 hr after the injection, the liver was sampled and subjected to assay for radioactivity. Differences in the amounts of ^{111}In-PLGA accumulated in the liver were statistically evaluated by Student's *t*-test.

Pharmacokinetic Analysis

Tissue distribution patterns of ^{111}In-labeled PLGA derivatives were evaluated from the organ uptake clearance (CL_{org}) according to the method of Takakura et al. (2). In the early period after injection, the efflux of ^{111}In radioactivity from organs is assumed to be negligible since the degradation products of ^{111}In-labeled ligands using DTPA cannot easily pass through biological membranes (12). With the assumption described above, CL_{org} can be calculated by dividing the amount of radioactivity in an organ at an appropriate interval of time by the area under the plasma concentration-time curve (AUC) up to the same time point. AUC and the total-body clearance (CL_{total}) were calculated by fitting an equation derived from a linear one- or two-compartment open model to the plasma concentration data of ^{111}In-labeled derivative using the nonlinear least-square program MULTI (13).

Degradation of ^{111}In-labeled Gal-PLGA in the Liver

Mice were injected with ^{111}In-labeled Gal-PLGA in saline at a dose of 1 mg/kg. At 10, 30 min and 6 hr after injection, mice were killed and the liver was excised. The liver was homogenized with 5 ml of distilled water and centrifuged at 20,000 rpm for 30 min at 4°C. The supernatant of the liver homogenate was applied to a Sephadex G-50 column (1 × 40 cm) and eluted with 0.1 M acetate buffer (pH 6.0) at a rate of 1 ml/min, and the radioactivity of each fraction was counted. The degraded fraction was calculated from the elution pattern.

RESULTS

Physicochemical Characteristics of PLGA Derivatives

Table I summarizes the physicochemical properties of the six types of galactosylated derivatives of PLGA synthesized. The apparent molecular weights of the derivatives were essentially unchanged by the attachment of galactose to PLGA. The average numbers of galactose residues per PLGA molecule were calculated from 3.7 to 19.5.

Plasma Clearance and Tissue Distribution of ^{111}In-PLGA and ^{111}In-Gal-PLGAs

Fig. 1 shows the concentration in plasma and the amounts in the liver, kidneys and urine of ^{111}In-PLGA after intravenous injection in mice. ^{111}In-PLGA was rapidly cleared from the circulation and predominantly accumulated in the kidneys. The amount recovered in the liver was about 15% of the dose, which was about half of that in the kidneys. The other tissues had no significant radioactivity (data not shown).

On the other hand, ^{111}In-Gal-PLGAs rapidly disappeared from the plasma and taken up by the liver (Fig. 2). The elimination rate from plasma and the uptake rate by the liver were highly dependent on the number of galactose residues per PLGA molecule; the maximum amounts recovered in the liver were 6.7, 16, 28, 61, 72 and 91% of the dose for ^{111}In-$Gal_{3.7}$-PLGA, $Gal_{4.4}$-PLGA, $Gal_{6.6}$-PLGA, Gal_{16}-PLGA, Gal_{18}-PLGA and Gal_{20}-PLGA, respectively. The amounts of ^{111}In-$Gal_{3.7}$-PLGA recovered in the liver was lower than that of ^{111}In-PLGA.

Table I. Physicochemical Characteristics of PLGA and Its Galactosylated Derivates (Gal-PLGA)

Compounds	Apparent[a] molecular weight	Number of[b] galactose residues (mol/mol PLGA)	Galactose[b] content (%)
PLGA	25200	—	—
$Gal_{3.7}$-PLGA	N.D.[c]	3.71	2.58
$Gal_{4.4}$-PLGA	26700	4.38	3.03
$Gal_{6.6}$-PLGA	26400	6.61	4.51
Gal_{16}-PLGA	26500	16.0	10.3
Gal_{18}-PLGA	25600	18.2	11.5
Gal_{20}-PLGA	N.D.	19.5	12.2

[a] Apparent molecular weights of PLGA and its derivatives were estimated by high-performance liquid chromatography gel filtration.
[b] The numbers of galactose residues and the galactose content of Gal-PLGAs were determined by anthrone-sulfuric acid method.
[c] N.D.: not determined.

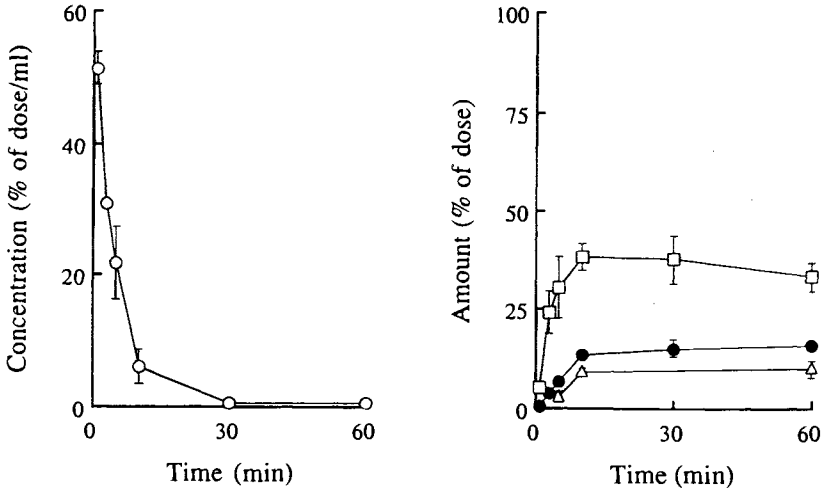

Fig. 1. Concentration in plasma (*left*) and amounts in the liver (●), kidneys (□) and urine (△) (*right*) of ^{111}In-PLGA after intravenous injection in mice at a dose of 1 mg/kg. Results are expressed as the mean ± SD of three mice.

Uptake of ^{111}In-PLGA and ^{111}In-Gal-PLGA by Liver Parenchymal and Nonparenchymal Cells

The contribution of liver-consisting cells to the hepatic uptake of ^{111}In-PLGA and ^{111}In-Gal_{18}-PLGA was determined by fractionating liver cells into PC and NPC by collagenase perfusion. After the injection of ^{111}In-PLGA, 1.2 and 2.4% of the dose were recovered in 10^8 cells of PC and NPC, respectively. On the other hand, after the administration of ^{111}In-Gal_{18}-PLGA, 35% of the dose per 10^8 cells was predominantly detected in PC, whereas only 0.3% of the dose was counted in NPC.

The hepatic uptake of ^{111}In-PLGA at a dose of 1 mg/kg was reduced by the co-injection of maleylated and succinylated BSAs at a dose of 20 mg/kg, which are known to be taken up by liver nonparenchymal cells via the scavenger receptor (14). On the other hand, galactosylated or mannosylated BSA did not affect the amount of ^{111}In-PLGA recovered in the liver at 1 hr after the injection.

Pharmacokinetic Analysis of ^{111}In-labeled PLGA Derivatives

Table II summarizes the clearance values for liver (CL_{liver}), kidney (CL_{kidney}), urine (CL_{urine}), CL_{total} and AUC calculated from the results of *in vivo* distribution experiments. ^{111}In-PLGA had large CL_{kidney} and CL_{urine} values of 9.7 and 1.4 ml/hr, respectively. The total value of CL_{kidney} and CL_{urine} (11 ml/hr)

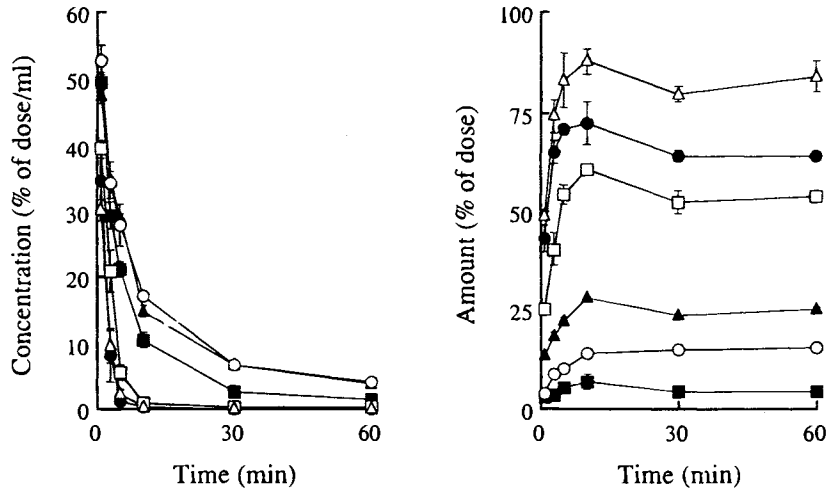

Fig. 2. Concentration in plasma (*left*) and amounts in the liver (*right*) of ^{111}In-Gal-PLGAs with different numbers of galactose residues after intravenous injection in mice at a dose of 1 mg/kg. Results are expressed as the mean ± SD of three mice. *Keys:* $Gal_{3.7}$-PLGA (■), $Gal_{4.4}$-PLGA (○), $Gal_{6.6}$-PLGA (▲), Gal_{16}-PLGA (□), Gal_{18}-PLGA (●) and Gal_{20}-PLGA (△).

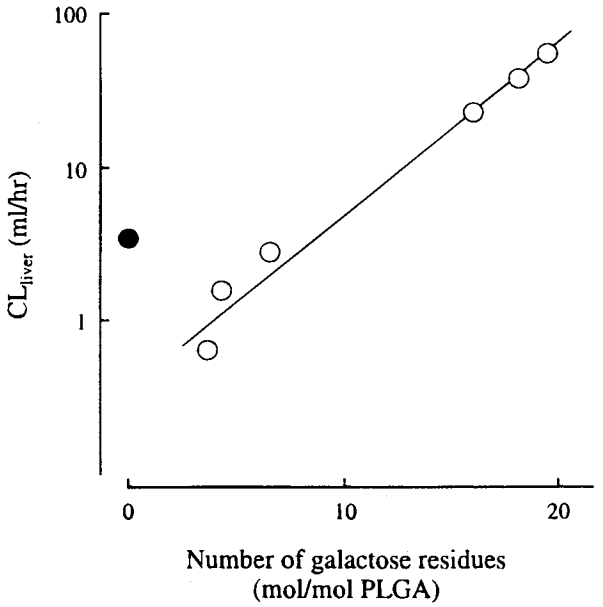

Fig. 3. Apparent hepatic uptake clearances of ^{111}In-PLGA and Gal-PLGAs having different numbers of galactose residues after intravenous injection in mice at a dose of 1 mg/kg.

was comparable to the glomerular filtration rate of mice (15). On the other hand, ^{111}In-Gal-PLGAs showed large CL_{liver} values highly depending on the number of galactose residues; e.g., ^{111}In-Gal_{20}-PLGA had about a 16-fold larger CL_{liver} value than ^{111}In-PLGA. However, ^{111}In-$Gal_{3.7}$-PLGA and $Gal_{4.4}$-PLGA had lower CL_{liver} values than ^{111}In-PLGA.

CL_{kidney} and CL_{urine} values of ^{111}In-Gal-PLGAs were also affected by the degree of galactose modification. The CL_{kidney} values decreased with an increase in the number of galactose residues per PLGA molecule, whereas the CL_{urine} value increased with the number of galactose residues.

Degradation of ^{111}In-labeled Gal_{18}-PLGA in the Liver

The molecular size of the radioactive products in the liver of mice injected with ^{111}In-Gal_{18}-PLGA was estimated by size-exclusion chromatography. More than 90% of the total radioactivity in the liver was recovered in the supernatant after centrifugation. While intact ^{111}In-Gal_{18}-PLGA was rapidly eluted from the column, low-molecular weight products appeared at 10 min after injection and the ratio of the degraded fraction was calculated to be about 20% of the total amount. The degraded fraction increased with time and more than 70% of ^{111}In-Gal_{18}-PLGA taken up by the liver was degraded within 30 min. At 6 hr after administration, about 90% of the radioactivity in the liver was recovered as low-molecular weight materials.

Table II. AUC and Clearances of ^{111}In-PLGA and Gal-PLGAs After Intravenous Injection in Mice at a Dose of 1 mg/kg

Compounds	AUC (% of dose hr/ml)	Clearance (ml/hr)			
		CL_{total}	CL_{liver}	CL_{kidney}	CL_{urine}
PLGA	4.68	21.3	3.45	9.66	1.44
$Gal_{3.7}$-PLGA	7.65	13.1	0.63	9.69	1.70
$Gal_{4.4}$-PLGA	10.2	9.79	1.55	5.44	2.26
$Gal_{6.6}$-PLGA	9.28	10.8	2.74	4.91	1.63
Gal_{16}-PLGA	2.40	41.6	22.5	5.36	2.93
Gal_{18}-PLGA	1.76	56.7	37.6	1.05	3.31
Gal_{20}-PLGA	1.53	65.5	55.1	0.71	3.14

DISCUSSION

In this study, PLGA and its derivatives were radiolabeled with ^{111}In using DTPA dianhydride as a bifunctional chelating agent since indium-DTPA chelate is reported to have an extremely high stability constant (16). In addition, after the ligands labeled with ^{111}In are degraded in the intracellular space, radioactive metabolites do not pass the plasma membrane and are retained in the cell (12). These features enable us not only to calculate CL_{org} by dividing the radioactivity in the organ by AUC up to the sampling point but also to trace the intracellular degradation of Gal-PLGA.

After the intravenous injection, ^{111}In-PLGA rapidly disappeared from the plasma and was mainly recovered in the kidneys and urine. Since the molecular weight of PLGA (25 kDa) is smaller than the size of molecular sieving at the glomerulus (17), a large fraction of PLGA would be filtered at the glomerulus and reabsorbed at the proximal tubules in the kidney. On the other hand, approximately 15% of the dose was recovered in the liver; this resulted in a relatively high CL_{liver} value (3.4 ml/hr) compared to those of ^{111}In-labeled unmodified proteins such as BSA, bovine γ-immunogloblins, and recombinant human superoxide dismutase (4). Polyanionic macromolecules such as poly[I] and succinylated or maleylated proteins are known to be recognized by the scavenger receptor and to be taken up by the liver after intravenous administration (14). In the present study, ^{111}In-PLGA was preferentially recovered in NPC and simultaneous injections with maleylated and succinylated BSA reduced its hepatic uptake. On the other hand, co-injection with galactosylated or mannosylated BSA did not affect the hepatic uptake of ^{111}In-PLGA. These findings suggest that PLGA is recognized and taken up by liver nonparenchymal cells via the scavenger receptor-mediated endocytosis.

Attachment of galactose moieties endowed PLGA with the targeting property to the liver. In addition, the amounts delivered to the liver highly depended on the number of galactose residues per PLGA molecule. In our previous study using globular proteins (5), we found that the density of galactose residues on the surface of protein molecules is the main determinant of the affinity of galactosylated proteins to the asialoglycoprotein receptor. The present findings suggest that Gal-PLGA is also recognized by the receptor depending on the number or the density of galactose residues.

Pharmacokinetic analysis further clarified the importance of the number of galactose residues. The CL_{liver} values of ^{111}In-Gal-PLGAs were highly dependent on the number of galactose residues per PLGA molecule; i.e., the CL_{liver} value of ^{111}In-Gal_{20}-PLGA was about 55 ml/hr which is comparable to the hepatic plasma flow rate. On the other hand, the CL_{liver} values of ^{111}In-$Gal_{3.7}$-PLGA and $Gal_{4.4}$-PLGA were lower than that of ^{111}In-PLGA, suggesting that the partial loss of negative charges of PLGA with galactosylation would overcome the increase in the affinity due to galactose attachment. Galactose modification also affected the CL_{kidney} and CL_{urine} of ^{111}In-PLGA derivatives,

probably because attachment of galactose moieties to PLGA inhibits the reabsorption of derivatives at the proximal tubules in the kidneys after the glomerular filtration.

As we reported previously, the hepatic uptake of galactosylated macromolecules depends on the administered dose since a higher concentration of galactosylated ligands will cause the saturation of the asialoglycoprotein receptor-mediated uptake. Since Gal-PLGAs have relatively large extrahepatic elimination clearances, their dose should be controlled to achieve an efficient hepatic delivery of drugs using Gal-PLGAs as hepatotropic carriers. The use of a derivative of PLGA with a higher molecular weight would increase the amounts of drugs delivered to the liver. In addition, intrinsic asialoglycoproteins could also inhibit the hepatic uptake of galactosylated macromolecular carriers. However, synthesized ligands reportedly possess higher affinity to the receptor than asialoorosomucoid, an intrinsic asialoglycoprotein (18).

Ligands taken up by receptor-mediated endocytosis are generally delivered to endosomes and lysosomes and are degraded in these compartments. PLGA is also reported to be degraded by proteolytic enzymes and is labile in the lysosome compartment (19). In general, the macromolecule used as the drug carrier should be stable in blood circulation and be degraded after delivering the drug to the target site. While Gal-PLGAs were not degraded in mouse plasma for 1 hr (data not shown), more than 70% of ^{111}In-Gal$_{18}$-PLGA taken up by the liver was degraded within 30 min. This indicates that Gal-PLGAs maintain the biodegradable property of PLGA even after chemical modification, which is advantageous for efficient drug release from the carrier in target cells.

In conclusion, we developed a new drug carrier system using PLGA for liver-specific targeting. PLGA derivatives with many galactose residues were efficiently delivered to liver parenchymal cells and easily degraded in these cells. In a preliminary study, we succeeded to target vitamin K_5, a coagulant, to the liver using Gal-PLGA and obtained enhanced antihemorrhagic activity against hemorrhagic mice (unpublished results).

ACKNOWLEDGMENTS

This work was supported in part by a grant for "Basic Research on Drug Innovation" by Japan Health Science Foundation.

REFERENCES

1. Y. Takakura and M. Hashida. Macromolecular drug carrier systems in cancer chemotherapy: Macromolecular prodrugs. *Crit. Rev. Oncol. Hematol.* **18**:207–231 (1995).
2. Y. Takakura, A. Takagi, M. Hashida and H. Sezaki. Disposition and tumour localization of mitomycin C-dextran conjugates in mice. *Pharm. Res.* **4**:293–300 (1987).
3. M. Hashida and Y. Takakura. Pharmacokinetics in design of polymeric drug delivery systems. *J. Controlled Release* **31**:163–171 (1994).
4. M. Nishikawa, H. Hirabayashi, Y. Takakura and M. Hashida. Design for cell-specific targeting of proteins utilizing sugar-recognition mechanism: Effect of molecular weight of proteins on targeting efficiency. *Pharm. Res.* **12**:209–214 (1995).
5. M. Nishikawa, C. Miyazaki, F. Yamashita, Y. Takakura and M. Hashida. Galactosylated proteins are recognized by the liver according to the surface density of galactose moieties. *Am. J. Physiol.* **268**:G849–G856 (1995).
6. M. Nishikawa, A. Kamijo, T. Fujita, Y. Takakura, H. Sezaki and M. Hashida. Synthesis and pharmacokinetics of a new liver-specific carrier, glycosylated carboxymethyl-dextran, and its application to drug targeting. *Pharm. Res.* **10**:1253–1261 (1993).
7. T. Fujita, M. Nishikawa, C. Tamaki, Y. Takakura, M. Hashida and H. Sezaki. Targeted delivery of human recombinant superoxide dismutase by chemical modification with mono- and poly-saccharide derivatives. *J. Pharmacol. Exp. Ther.* **263**:971–978 (1992).
8. T. Fujita, M. Nishikawa, C. Tamaki, Y. Takakura, M. Hashida and H. Sezaki. Therapeutic effects of superoxide dismutase derivatives modified with mono- or polysaccharides on hepatic injury induced by ischemia/reperfusion. *Biochem. Biophys. Res. Commun.* **189**:191–196 (1992).
9. C. F. Roos, S. Matsumoto, Y. Takakura, M. Hashida and H. Sezaki. Physicochemical and antitumor characteristics of some polyamino acid prodrugs of mitomycin C. *Int. J. Pharm.* **22**:75–87 (1984).
10. Y. Morimoto, K. Sugibayashi, S. Sugihara, K. Hosoya, S. Nozaki and Y. Ogawa. Antitumor agent poly (amino acid) conjugates as a drug carrier in cancer chemotherapy. *J. Pharmacobio-Dyn.* **7**:688–698 (1984).
11. K. Nishida, C. Tonegawa, S. Nakane, Y. Takakura, M. Hashida and H. Sezaki. Effect of electric charge on the hepatic uptake of macromolecules in the rat liver. *Int. J. Pharm.* **65**:7–17 (1990).
12. J. R. Duncan and M. J. Welch. Intracellular metabolism of indium-^{111}-DTPA-labeled receptor targeted proteins. *J. Nucl. Med.* **34**:1728–1738 (1993).
13. K. Yamaoka, Y. Tanigawara, H. Tanaka and Y. Uno. A pharmacokinetic analysis program (MULTI) for microcomputer. *J. Pharmacobio-Dyn.* **4**:879–885 (1981).
14. Y. Takakura, T. Fujita, H. Furitsu, M. Nishikawa, H. Sezaki and M. Hashida. Pharmacokinetics of succinylated proteins and dextran sulfate in mice: Implication for hepatic targeting of protein drugs by direct succinylation via scavenger receptors. *Int. J. Pharm.* **105**:19–29 (1994).
15. K. B. Bischoff, R. L. Dedrick, D. S. Zaharko and J. A. Longstreth. Methotrexate pharmacokinetics. *J. Pharm. Sci.* **60**:1128–1133 (1971).
16. K. M. Subramanian and W. Wolf. A new radiochemical method to determine the stability constants of metal chelates attached to a protein. *J. Nucl. Med.* **31**:480–488 (1990).
17. B. M. Brenner, T. H. Hostetter and H. D. Humes. Glomerular permselectivity: Barrier function based on discrimination of molecular size and charge. *Am. J. Physiol.* **234**:F455–F460 (1978).
18. M. J. Krantz, N. A. Holtzman, C. P. Stowell and Y. C. Lee. Attachment of thioglycosides to proteins: Enhancement of liver membrane binding. *Biochemistry* **15**:3963–3968 (1976).
19. R. B. Hawkins and A. Holtzer. Some macromolecular properties of poly (α-L-glutamic acid) random coils. *Macromolecules* **5**:294–301 (1972).