Disposition Characteristics of Model Macromolecules in the Perfused Rat Kidney

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The disposition characteristics of model macromolecules such as dextran (70 kDa), bovine serum albumin (BSA), and their charged derivatives were studied in the perfused rat kidney. In a single-pass indicator dilution experiment, venous and urinary recovery patterns and tissue accumulation of radiolabeled compounds were evaluated under filtering or nonfiltering conditions. In the filtering kidney, cationic macromolecules such as diethylaminoethyl-dextran (DEAE-dex) and cationized BSA (cBSA) accumulated in the kidney to a great extent whereas anionic and neutral macromolecules such as BSA, carboxymethyl-dextran (CM-dex), and dextran showed only small uptake. DEAE-dex and cBSA were distributed to both the medulla and cortex regions of the kidney and their recoveries in the kidney decreased as the injected dose increased. Similar tissue uptake was observed in the nonfiltering kidney perfusion system suggesting that they were mainly taken up by the kidney from the renal capillary side based on electrostatic interaction. In addition, the steady-state distribution volumes of cationic macromolecules calculated from venous outflow patterns were larger than those of the intravascular volume estimated from the distribution volumes of neutral and anionic macromolecules, suggesting their reversible interaction with the vascular wall. On the other hand, dextran derivatives with molecular weight distribution were excreted into urine based on glomerular permselectivity; *i.e.*, cationic DEAE-dex and anionic CM-dex showed enhanced and restricted urinary excretion, respectively, compared with neutral dextran. In contrast, no significant excretion was observed for BSA and cBSA. The utility of the isolated rat kidney perfusion experiment for studying the renal disposition of macromolecular drugs was thus demonstrated.

Keywords macromolecule; rat kidney perfusion experiment; indicator dilution; glomerular filtration; peritubular uptake; tubular reabsorption

Introduction

Recently, various macromolecular drug carrier systems have been developed in order to control the *in vivo* behavior of drugs.¹⁾ However, their application has often been limited since their basic disposition profiles are strictly decided by their renal and hepatic excretion or uptake. In our series of investigations, we have examined the *in vivo* behavior of model macromolecules and polymeric prodrugs of mitomycin C in rats and mice.²⁾ Simply speaking, cationic macromolecules accumulated in the liver and kidney to a great extent, while weakly anionic and electrically neutral macromolecules showed very small accumulation. The liver perfusion experiments³⁾ demonstrated the actual status of hepatic uptake of cationic macromolecules and gave the numbers of binding sites and dissociation constants for interaction due to electrostatic force and internalization constants.

The mechanism of renal accumulation of these macromolecules, however, has only been investigated quantitatively, although some investigations have phenomenologically reported about glomerular permselectivity for dextrans and low molecular weight proteins.⁴⁾ In general, renal accumulation of macromolecules is due to tubular reabsorption following glomerular filtration as shown in small proteins such as lysozyme, cytochrome, and growth hormone,⁵⁾ or to receptor mediated endocytosis from the vascular side as shown in hormonal proteins such as insulin, calcitonin and epidermal growth factor.⁶⁾

In the present study, we analyzed the disposition characteristics of model macromolecules which possess similar molecular weights but different electrical charges using

a perfused rat kidney under filtering and nonfiltering conditions.

Materials and Methods

Chemicals Dextran(T-70) with an average molecular weight of 70 kDa was obtained from Pharmacia, Uppsala, Sweden. Bovine serum albumin (BSA) was obtained from Armour Pharmaceutical Co., U. K. [¹¹¹In]Cl₃ (74 MBq/ml) was supplied by Nihon Medipysics, Co., Takarazuka, Japan. Potassium[¹⁴C]cyanide (30 MBq/mg) was supplied from Amersham Japan (Tokyo, Japan). [Methoxy-¹⁴C]inulin (185 MBq/g) was purchased from New England Nuclear (Boston, MA). All other chemicals were reagent grade products obtained commercially.

Syntheses of Model Macromolecules Diethylaminoethyl-dextran (DEAE-dex) and carboxymethyl-dextran (CM-dex) were synthesized according to the method of McKernan *et al.*⁷⁾ and Peterson *et al.*,⁸⁾ respectively. Cationized bovine serum albumin (cBSA) was synthesized by covalent coupling of hexamethylenediamine to BSA.⁹⁾ The product was purified by chromatofocusing using Polybuffer exchanger 94 resin and the Polybuffer 96 elution buffer system (Pharmacia, Uppsala, Sweden). The major protein peak eluted in the region of pI > 9.0 was collected. The modified macromolecules had almost the same effective molecular size as the original dextran(T-70) and BSA on gel-filtration chromatography.

Radiolabeling of Model Macromolecules [Carboxy-¹⁴C]dextran(T-70) was prepared using potassium [¹⁴C]cyanide according to the method of Isbell *et al.*¹⁰⁾ with slight modification. Radiolabeled DEAE-dex(T-70) and CM-dex(T-70) were synthesized from ¹⁴C-dextran in the same manner as the unlabeled dextrans. BSA and cBSA were radiolabeled with ¹¹¹In using a bifunctional chelating agent, diethylene-triaminepentaacetic acid (DTPA) anhydride (Dojindo Labs, Kumamoto, Japan) according to the method of Hnatowich *et al.*¹¹⁾

Perfusate In filtering and nonfiltering kidney perfusion experiments, the perfusate consisted of Krebs-Henseleit bicarbonate buffer, pH 7.45, containing glucose (5 mM) and BSA (filtering kidney: 5%, nonfiltering: 10%). The perfusate was normally gassed with 95% O₂/5% CO₂.

Isolated Rat Kidney Perfusion The kidneys of male Wistar rats weighing 210–240 g were isolated by the method of Nishiitsutsuji-Uwo *et al.*¹²⁾ Briefly, rats were anesthetized with pentobarbital (40 mg/kg, i.p.) and the

femoral vein was exposed, to which 100 mg (1 ml) of mannitol was administered. After making a midline incision, the right kidney was exposed and the ureter was cannulated for urine collection by polyethylene (PE-10) tubing. A venous catheter (o.d. 2.33 mm, i.d. 1.33 mm) was placed in the *vena cava* and the renal artery was cannulated using a 20 G needle *via* the mesenteric artery to avoid interrupting the renal blood flow. The whole operation took about 15 min. Immediately after cannulation, the kidney was isolated from the animal and perfused at a rate of 18 ml/min for 20 min to obtain a stable condition. The renal arterial pressure was monitored through the experiment and maintained at 70–80 mmHg by adjusting the perfusate flow rate. The nonfiltering kidney experiment was accomplished by tying off the ureters, raising the perfusate albumin concentration to 10%, and lowering the renal arterial pressure to 55 mmHg.¹³⁾

Bolus Injection Experiment Saline (0.14 ml) dissolving BSA (5%) and test macromolecules were introduced into the arterial catheter using a six-position rotary valve injector (Type 50 Teflon rotary valves, Rheodyne, Cotati, CA.). Venous outflow samples were collected into the previously weighed tube at 0.3- to 3-s intervals for 20 s. The collected sample volumes were calculated from the gain in weight of the tube assuming the density of the outflowing perfusate to be 1.0. The mean sampling time point was estimated from each sample volume assuming a constant flow rate. Urine samples were collected into the weighed tubes for 14 min. The sampling interval was 1 min at first and was gradually prolonged. The urine volumes were calculated in the same way as the venous outflow samples. After finishing the perfusion experiment, the wet weight of excised kidney was measured and slices of renal cortex and medulla were obtained for assay.

Assay ¹⁴C radioactivity was determined by a liquid scintillation counter (LSC-5000, Beckman, Tokyo, Japan). Perfusate sample (0.1 ml) was added to a vial with 5 ml of scintillation medium (Clear-sol I, Nacalai Tesque, Kyoto, Japan). The urine and kidney tissues were put into a counting vial with 0.7 ml of Soluene-350 (Packard Instrument, CT). After incubation at 45 °C for 10 h, 5 ml of scintillation medium was added. The ¹¹¹In radioactivity was measured by a well NaI scintillation counter (ARC-500, Aloka, Tokyo, Japan) without any special procedure.

Data Analysis The statistical moment parameters for the outflow pattern are calculated as follows:

$$AUC = \int_0^{\infty} C dt$$

$$MTT_{kid} = \int_0^{\infty} t C dt / AUC$$

where t is time and C represents the concentration of compounds normalized by the injection dose. AUC and MTT_{kid} denote the area under the concentration–time curve and mean transit time of the drug in the kidney, respectively. These parameters are calculated by numeric integration using a linear trapezoidal formula and extrapolation to infinite time based on a single-exponential equation.¹⁴⁾ The steady-state distribution volume is calculated from the moment parameters as follows:

$$F_o = AUC Q$$

$$V_d = Q MTT_{kid} / F_o / \text{kidney weight}$$

where F_o corresponds to the venous outflow recovery, Q represents the perfusion flow rate and V_d is the steady-state distribution volume normalized by kidney weight.

The urinary excretion rate–time curve is also analyzed based on statistical moment theory as follows:

$$F_u = \int_0^{\infty} (dX_u/dt) dt$$

$$MTT_u = \int_0^{\infty} t (dX_u/dt) dt / F_u$$

where dX_u/dt is the urinary excretion rate normalized with the injection dose and expressed in a percentage of the dose per minute. MTT_u is the urinary mean excretion time.

Results

Isolated Rat Kidney Perfusion In this perfusion system, the average kidney weight was 1.06 ± 0.11 (S.D.) g. Plasma flow rate (PFR) (17.1 ± 2.8 ml/min), glomerular filtration

Fig. 1. Typical Venous Outflow Curves of Model Macromolecules after Bolus Injection in the Rat Kidney Perfusion Experiment

A: ●, ¹⁴C-dextran (T-70); △, ¹⁴C-DEAE-dex (T-70); □, ¹⁴C-CM-dex (T-70). B: ▲, ¹¹¹In-BSA; ■, ¹¹¹In-cBSA; ○, ¹⁴C-inulin.

rate (GFR) (0.41 ± 0.10 ml/min), perfusion pressure (76 ± 9 mmHg), urine flow rate ($50\text{--}100$ μl/min), and glucose reabsorption ratio ($>90\%$) were maintained at almost constant values, which were comparable with those reported in other studies.¹⁵⁾ Although erythrocytes were not added to the perfusate and the PFR was 3–4 times larger than normal, kidney functions seemed to be maintained.¹⁶⁾

Venous Recovery of Macromolecules Figure 1 illustrates typical outflow concentration–time curves of 6 model macromolecules after bolus injection. In all cases, more than 90–95% of the injected dose was recovered in the venous outflow during the first 10 s after injection. Dextran(T-70), CM-dex(T-70), and BSA showed almost the same outflow patterns with pointed peaks. By comparison, the peak of inulin was gentle. cBSA showed a slower appearance and lower peak value. The moment parameters and steady-state distribution volume (V_d) for model macromolecules at different doses were calculated from these outflow patterns as summarized in Table I. The V_d of inulin which corresponds to the extracellular volume of the experimental system was determined to be 0.45 ml/g. BSA showed the smallest V_d of about 0.25 ml/g which corresponds to the intravascular volume.¹⁷⁾ Anionic CM-dex(T-70) showed almost identical values to that of BSA while cationic cBSA and DEAE-dex(T-70) gave higher values than that of BSA. At higher doses, V_d values of cBSA and DEAE-dex(T-70) significantly decreased.

Urinary Excretion of Macromolecules Figure 2 shows the typical urinary excretion rate–time curves of six model macromolecules. All test substances were excreted in urine in intact forms within 14 min after injection. Dextran derivatives were excreted in urine and the profile varied between derivatives. On the other hand, significant amounts of BSA and cBSA were not detected in urine. Table I summarizes the calculated moment parameters, and similar values of MTT_u were obtained for all model

TABLE I. Moments and Distribution Volumes of Model Macromolecules in the Rat Kidney Perfusion Experiments

	Dose (mg/kidney)	AUC (% of dose s/ml)	MTT _{kid} (s)	V _d (ml/g kidney)	MTT _u (min)
¹⁴ C-Dextran (T-70)	0.14	354.0 ± 15.6	1.150 ± 0.041	0.283 ± 0.012	2.244 ± 0.363
	1.4	339.0 ± 82.2	1.042 ± 0.140	0.295 ± 0.016	1.631 ± 0.325
¹⁴ C-DEAE-dex (T-70)	0.14	312.4 ± 29.9	1.075 ± 0.072	0.372 ± 0.026	2.286 ± 0.219
	1.4	343.0 ± 7.1	1.090 ± 0.062	0.303 ± 0.006	2.076 ± 0.366
¹⁴ C-CM-dex (T-70)	0.14	309.4 ± 39.6	0.948 ± 0.063	0.279 ± 0.003	1.902 ± 0.055
	1.4	329.3 ± 19.8	0.958 ± 0.139	0.252 ± 0.033	1.619 ± 0.080
¹¹¹ In-BSA		325.6 ± 32.3	0.924 ± 0.149	0.254 ± 0.026	N.D.
¹¹¹ In-cBSA	0.014	343.1 ± 37.4	1.289 ± 0.118	0.389 ± 0.009	N.D.
	0.14	316.6 ± 20.8	0.994 ± 0.009	0.282 ± 0.000	N.D.
	1.4	340.5 ± 16.0	0.879 ± 0.079	0.250 ± 0.005	N.D.
¹⁴ C-Inulin	0.01	352.3 ± 48.8	1.595 ± 0.124	0.449 ± 0.021	1.648 ± 0.454

Results are expressed as the mean ± S.D. of at least three experiments. N.D.: Not determined.

Fig. 2. Typical Urinary Excretion Rate-Time Curves of Model Macromolecules after Bolus Injection in the Rat Kidney Perfusion Experiment

A: ●, ¹⁴C-dextran (T-70); △, ¹⁴C-DEAE-dex (T-70); □, ¹⁴C-CM-dex (T-70). B: ▲, ¹¹¹In-BSA; ■, ¹¹¹In-cBSA; ○, ¹⁴C-inulin.

macromolecules including inulin.

Renal Accumulation of Macromolecules Table II shows the amount of recovery of radioactivity in the kidney and in the total urine at 14 min after bolus injection of model macromolecules at different doses. The amount of recovered cBSA and DEAE-dex(T-70) in the kidney was larger than that of other macromolecules and decreased with an increase in the dose. On the other hand, the amounts of dextran derivatives recovered in the urine were independent of the dose.

Nonfiltering Kidney Perfusion Experiments In Table II, the amount of recovered DEAE-dex(T-70), cBSA, and inulin in the kidney and urine at a dose of 0.14 mg/kidney in the nonfiltering and filtering kidney perfusion experiments was compared. The amount of recovered cationic macromolecules in the filtering and nonfiltering kidneys were almost similar to each other. In both experimental systems, these cationic macromolecules were distributed to the renal cortex and medulla regions of tissue as shown

TABLE II. Amount of Recovery in the Kidney and Urine of Six Macromolecules at 14 min after Injection in the Filtering and Nonfiltering Kidney Perfusion Experiments

	Dose (mg/kidney)	Kidney (% of dose)	Urine (% of dose)	Total (% of dose)
¹⁴ C-Dextran (T-70)	0.14	0.166 ± 0.034	0.375 ± 0.062	0.541 ± 0.029
	1.4	0.082 ± 0.047	0.417 ± 0.087	0.499 ± 0.119
¹⁴ C-DEAE-dex (T-70)	0.14	3.088 ± 0.204	0.960 ± 0.178	4.047 ± 0.029
	1.4	1.978 ± 0.226	1.022 ± 0.243	3.001 ± 0.154
Nonfiltering	0.14	2.756 ± 0.149	N.D.	2.756 ± 0.149
¹⁴ C-CM-dex (T-70)	0.14	0.087 ± 0.004	0.321 ± 0.099	0.407 ± 0.103
	1.4	0.094 ± 0.016	0.292 ± 0.010	0.386 ± 0.026
¹¹¹ In-BSA		0.054 ± 0.040	0.033 ± 0.025	0.087 ± 0.030
¹¹¹ In-cBSA	0.014	8.38 ± 1.729	0.043 ± 0.011	8.422 ± 1.740
	0.14	4.226 ± 0.620	0.032 ± 0.011	4.258 ± 0.630
	1.4	1.757 ± 0.275	0.024 ± 0.011	1.781 ± 0.284
Nonfiltering	0.14	3.901 ± 1.557	N.D.	3.901 ± 1.557
¹⁴ C-Inulin	0.01	0.229 ± 0.020	2.393 ± 0.468	2.622 ± 0.481
Nonfiltering	0.01	0.148 ± 0.010	N.D.	0.148 ± 0.010

Results are expressed as the mean ± S.D. of at least three experiments.

Fig. 3. Concentration of Cationic Macromolecules in the Kidney Cortex and Medulla at 14 min after Injection in the Kidney Perfusion Experiment

Results are expressed as the mean ± S.D. of at least three experiments. ■, cortex; □, medulla.

in Fig. 3. The concentration of DEAE-dex(T-70) in the medulla was lower than in the cortex, while cBSA showed relatively uniform distribution.

Discussion

Our series of investigations demonstrated an important role of the kidney in the disposition of macromolecules after i.v. injection.^{2c)} In these experiments, the electrical charge of the macromolecules showed a remarkable effect; i.e., cationic macromolecules accumulated in the kidney

to a great extent while anionic ones did not. The present study was done to clarify the urinary disposition characteristics of macromolecules in relation to their physicochemical properties using perfused rat kidney. Several model macromolecules with similar molecular weights but different charges were used for the assessment of the effect of electric charge without any effect of metabolic degradation. That is, ^{14}C -labeled dextran resists degradation in organs,¹⁸⁾ and ^{111}In labeled to BSA derivatives accumulate in the tissue by being exchanged into an iron-binding protein after intracellular degradation.¹⁹⁾

The kidney perfusion experiment allowed us to elucidate the total renal disposition profiles of macromolecules. The marked and non-linear accumulation of cationic macromolecules, DEAE-dex(T-70) and cBSA, in the kidney suggested that they were taken up by the tissue based on saturable electrostatic interaction. At low doses, these accumulation amounts were larger than the total urinary recovery of inulin, a marker substance of GFR (Table II). When renal accumulation of macromolecules occurred only through reabsorption at the renal brush border membrane after glomerular filtration, the accumulation amount could not exceed the excreted amount of inulin. Therefore, cationic macromolecules were suggested to be taken up not only from the luminal side of renal tubular cells but also from the glomerulus and postglomerular capillary side. Actually, the results of the nonfiltering kidney experiments (Table II) suggested the fact that the uptake of cationic macromolecules occurred on the capillary side, since the recovered amount was not changed under the condition without glomerular filtration. In addition, a significant level of cationic macromolecules was observed in both the cortex and medulla (Fig. 3). These amounts in the filtering perfused kidney were essentially identical to those in the nonfiltering perfused kidney,

which also suggested that these cationic macromolecules were taken up mainly from the capillary side. This is contrary to the intra-renal distribution patterns of superoxide dismutase and soybean trypsin inhibitor which was restricted in the cortex due to predominant uptake from the luminal side (unpublished). These results correspond well to the findings that polycations associate with the glomerulus²⁰⁾ and tubular basement membrane²¹⁾ and support the conclusion that the cationic macromolecules accumulated from the vascular side. Although polycations were reported to be internalized *via* adsorptive endocytosis in brain capillaries,⁹⁾ the uptake mechanism in the kidney at the cellular level remains to be elucidated.

On the other hand, the steady-state distribution volumes (V_d) of DEAE-dex(T-70) and cBSA were larger than those of the neutral and anionic macromolecules (Table I), although they decreased as the dose increased and reached values almost similar to those of dextran(T-70) and BSA, respectively, at the highest dose (1.4 mg/kidney). These results suggested the existence of a reversible and saturable interaction of cationic macromolecules with the renal capillary wall. The difference in mechanism of reversible interaction and irreversible uptake is obscure.³⁾

The glomerular capillary wall is known to function as a size and charge selective barrier in glomerular filtration.⁴⁾ In the present investigation, dextran and its derivatives have been used as test macromolecules since they have consecutive molecular size distribution and are not very susceptible to tubular reabsorption. As shown in Fig. 2, the urinary recovery of DEAE-dex(T-70) was highest, and that of neutral dextran(T-70) followed this, reflecting a charge selectivity of the glomerulus.^{4a)} Linearity of glomerular filtration was also confirmed. In addition, all MTT_u values were comparable to those of inulin, suggesting their similar behaviors in the luminal space

Fig. 4. Renal Disposition Profiles of Model Macromolecules in the Kidney Perfusion Experiment

a) Non-linear process. The glomerular filtration amount was calculated by subtraction of the amount recovered in the nonfiltering kidney from the total amount recovered from the filtering kidney and urine. The tubular reabsorption amount was calculated by subtracting of the amount recovered from urine from the glomerular filtration amount.

after glomerular filtration. In contrast to dextrans with consecutive molecular size distribution whose smaller fraction could pass through the sieving pores of the glomerular wall, BSA and cBSA were not detected in urine, probably because their molecules were larger than the pores. The participation of tubular reabsorption⁵⁾ was negated in this case since similar uptake was observed in the nonfiltering kidney.

Figure 4 schematically summarizes the renal disposition properties of six model macromolecules. In this diagram, their glomerular filtration, tubular reabsorption, and vascular side uptake processes are macroscopically but quantitatively estimated. Thus, the present experimental system using perfused rat kidney was demonstrated to be useful for quantitative study of the renal disposition of macromolecules under various conditions. We will apply this system to elucidate the renal disposition of various kinds of polymeric prodrugs and protein derivatives in following papers.

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