Characterization of LLC-PK$_1$ Kidney Epithelial Cells as an *in Vitro* Model for Studying Renal Tubular Reabsorption of Protein Drugs

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Purpose. The purpose of this study was to assess whether LLC-PK$_1$ renal epithelial cells could serve as an *in vitro* model for studying the renal tubular reabsorption of protein drugs.

Methods. The association of ^{111}In-labeled model protein drugs, bovine serum albumin (BSA), superoxide dismutase (SOD), soybean trypsin inhibitor (STI), and [Asu^{1,7}]-eel calcitonin (Asu-ECT), with the monolayers of LLC-PK$_1$ renal epithelial cells was characterized under various conditions.

Results. The cellular association of these proteins was temperature-dependent and varied according to the protein. Saturation kinetics were observed for STI association, with the apparent Km and Vmax values determined to be 66.3 μg/ml and 250 ng/mg protein/min, respectively. The association of STI decreased with increases in medium pH from 5.4 to 8.4 and was inhibited significantly by 2,4-dinitrophenol, sodium azide, cytochalasin B, and colchicine, suggesting that the cellular association involved endocytosis. Mutual inhibition was observed in competitive binding experiments with the four protein drugs, suggesting that they shared a common binding site on the luminal membrane of LLC-PK$_1$ cells. Taken together, these findings show that a variety of protein drugs bind to LLC-PK$_1$ cells in a non-specific manner and possibly undergo endocytosis, a phenomenon that is similar to *in vivo* proximal tubular reabsorption.

Conclusions. LLC-PK$_1$ renal epithelial cells would be a suitable model system for the study of the renal proximal tubular reabsorption of protein drugs.

KEY WORDS: LLC-PK$_1$ cells; renal protein reabsorption; protein drugs; adsorptive endocytosis; chemical modification of protein drug.

INTRODUCTION

With the recent great progress in biotechnology, a variety of peptides and proteins have formed a new class of therapeutic agents. However, their clinical application is often limited by their rapid *in vivo* clearance. The kidney plays an important role in the disposition of protein drugs, since proteins with a molecular mass of less than 30 kDa are susceptible to glomerular filtration and undergo metabolic degradation in this organ (1,2). To optimize protein drug delivery, it is necessary to understand and control the renal handling of proteins.

We have previously characterized the renal disposition of model protein drugs in the isolated perfused rat kidney (3). That experimental system enabled us to evaluate their renal disposition processes, such as glomerular filtration, tubular reabsorption, and uptake from the capillary side, in a quantitative manner at the organ level. Based on those findings, we developed protein drug derivatives with desirable disposition and therapeutic characteristics in the kidney (4,5). However, further studies at the cellular and molecular levels are required for better understanding of the mechanism that underlies renal handling of protein drugs.

Studies of the renal handling of proteins have shown that the proximal tubule is the major site for the reabsorption of filtered proteins and that the initial step in reabsorption is endocytosis at the apical membrane (1,2). In the present study, we investigated the characteristics of protein drug association with monolayer cultures of LLC-PK$_1$ renal epithelial cells, which cells have biochemical and functional similarities to proximal tubule cells (6).

MATERIALS AND METHODS

Materials

[Methoxy-^{14}C]inulin (0.53 MBq/mmol) was obtained from New England Nuclear (Boston, Massachusetts). Indium chloride [$^{111}InCl_3$] was supplied by Nihon Mediphysics (Takarazuka, Japan). Bovine serum albumin (BSA; fraction V) and soybean trypsin inhibitor (STI) were obtained from Armour Pharmaceutical Co. (U.K.) and Sigma (St. Louis, Missouri), respectively. [Asu^{1,7}]-eel calcitonin (Asu-ECT) and superoxide dismutase (SOD) were kindly supplied by Asahi Kasei (Tokyo, Japan). All other chemicals were reagent grade products obtained commercially.

Synthesis of SOD Derivatives and Radiolabeling of Proteins

To examine the effect of chemical modification of protein drugs on their uptake by LLC-PK$_1$ cells, two SOD derivatives, cationized SOD (Cat-SOD) and mannosylated SOD (Man-SOD) were synthesized as reported previously (4). Table 1 summarizes the physicochemical properties of the protein drugs and SOD derivatives used in this study. Protein drugs and SOD derivatives were labeled with ^{111}In, using a bifunctional chelating agent, diethylenetriaminepentaacetic acid anhydride (Dojindo Labs, Kumamoto, Japan) (3,4). We used this radiolabeling method for evaluating the protein uptake process so as to exert minimal effects on metabolic degradation, since the radioactivity derived from ^{111}In-labeled proteins is accumulated in the cell after intracellular degradation (7).

Cell Culture

LLC-PK$_1$ cells (American Type Culture Collection, ATCC CRL1392) were maintained by serial passages in 25 cm^2 plastic culture flasks. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 %

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Table I. Physicochemical Properties Model Protein Drugs and SOD Derivatives

Protein	Number of NH_2	Molecular Weight	pI	Charge at pH 7.4
STI	12.0	20100	4.5–4.6	–
SOD	24.0	32000	5.0–5.2	–
BSA	60.0	67000	4.7–4.9[b]	–
Asu-ECT	3.0	3364	9.7	+
Cat-SOD	35.0[a]	34000	>9.0[b]	+
Man-SOD	3.8[a]	34000	n.d.	–

[a] Determined by trinitrobenzene sulfonic acid method.
[b] Determined by chromatofocusing method.
Calculated or cited value is used unless otherwise specified.

fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml), in an atmosphere of 5 % CO_2-95 % air at 37 °C. We subcultured the cells every 6-7 days, using 0.25 % trypsin-0.02 % EDTA solution, and plating the cells at a density of 4×10^4 cells/cm^2. For uptake experiments, 12-well dishes were inoculated with 7.2×10^4 cells in 1 ml of complete culture medium, and the cells were fed fresh medium on the 3th day after inoculation. The cells usually reached confluence 4-5 days after plating and the confluent cell monolayers were used for the uptake experiments. The cells were used between the 191st and 226th passages.

Cellular Association Experiments

The cellular association of protein drugs was examined with cultured LLC-PK$_1$ cells in 12-well dishes at 37 °C or 4 °C (on ice). After removal of the culture medium, each dish was washed twice with Hank's balanced salt solution (HBSS) (pH 7.4) buffer. HBSS buffer (0.75 ml), containing ^{111}In-labeled protein or ^{14}C-labeled polysaccharides, was then added to each dish, and the cells were allowed to incubate for a specified period at 37 °C or 4 °C. At the end of the incubation period, the buffer was immediately removed by aspiration. The cells were rapidly rinsed three times with 0.75 ml of ice-cold HBSS buffer and then solubilized in 0.75 ml of 0.3 N NaOH solution with 0.1 % Triton X-100. ^{111}In radioactivity was determined with a γ-scintillation counter (ARC-500; Aloka Co., Tokyo). ^{14}C radioactivity was determined with a liquid scintillation system (LSC-5000; Beckman, Tokyo, Japan) after the addition of 5 ml of scintillation medium (Clearsol I, Nacalai Tesque, Kyoto, Japan) to each sample. Protein content was determined by a modified Lowry method, with BSA as a standard.

RESULTS

Basic Characteristics of Protein Drug Association with LLC-PK$_1$ Cells

Figure 1 shows the association time courses of [^{111}In]-STI, [^{111}In]SOD, [^{111}In]BSA, [^{111}In]Asu-ECT and [^{14}C]inulin with the confluent LLC-PK$_1$ cell monolayer at 37 °C and 4 °C. The association rates of these protein drugs were significantly higher than that of [^{14}C]inulin, which was employed as the fluid-phase endocytosis marker. The association of [^{14}C]dextran was similar to that of [^{14}C]inulin (data not shown). Protein drug association was temperature-dependent and varied to a great extent depending on the protein.

Mechanism of Protein Drug Association with LLC-PK$_1$ Cells

To examine the mechanism underlying the protein drug association with LLC-PK$_1$ cells, the cellular association characteristics of [^{111}In]STI were studied under various conditions. Figure 2 shows the effects of the concentration of STI on its initial rate of association with LLC-PK$_1$ cells. Saturation kinetics were observed for the STI association, with the apparent Km and Vmax values determined to be 66.3 μg/ml and 250 ng/mg protein/min, respectively. On the other hand, [^{14}C]inulin association was not saturable within the same concentration range (data not shown). The effects of medium pH on [^{111}In]STI association are shown in Figure 3. The association decreased with increases in medium pH from 5.4 to 8.4. Figure 4 shows the effects of DNP, sodium azide (NaN_3), cytochalasin B, and colchicine on [^{111}In]STI association with LLC-PK$_1$ cells. The association was significantly inhibited in the presence of these metabolic and endocytosis inhibitors, suggesting that the protein drug association could be mediated by endocytosis.

Table 2 summarizes the effects of the other protein drugs on the binding of ^{111}In-labeled protein drugs at 4 °C. For [^{111}In]STI, [^{111}In]SOD, and [^{111}In]BSA, a significant binding inhibition was observed in the presence of excess amounts of the other cold proteins. On the other hand, [^{111}In]Asu-ECT binding was significantly inhibited by cold Asu-ECT but was not affected by STI and BSA.

Effects of Chemical Modification on Protein Drug Uptake by PK$_1$ Cells

SOD derivatives were employed to study the effects of chemical modification. The association of [^{111}In]Cat-SOD (14.2 ± 0.7 % of dose/mg protein/1.5 hr, n = 3) with LLC-PK$_1$ cells at 37 °C was significantly greater (* $p < 0.001$) than that of unmodified [^{111}In]SOD (2.4 ± 0.6 % of dose/mg protein/1.5 hr, n = 3) (data not shown), indicating that cationization of the protein drug greatly enhanced its cellular interaction. On the other hand, the value for [^{111}In]Man-SOD association (3.0± 0.1 % of dose/mg protein/4 hr, n = 3) was lower (* $p < 0.001$) than that for [^{111}In]SOD association (5.0 ± 0.1 % of dose/mg protein/4 hr, n = 3) (data not shown).

DISCUSSION

Cultured renal epithelial cells have been extensively employed as a useful model for studies of drug transport and drug effects in the proximal tubular cells (6). Schwegler *et al.* (8) have characterized confluent monolayers of the opossum kidney cell line (OK) as a model for proximal tubular protein reabsorption. With regard to LLC-PK$_1$ cells, although the characteristics of fluid phase and adsorptive endocytosis have been studied (9,10), little information is available about protein association characteristics in conjunction with the mechanism underlying protein reabsorption in the renal proximal tubules.

The apparent association rate of [^{14}C]inulin (5.7 μl medium/mg protein/60 min) in the present study was compara-

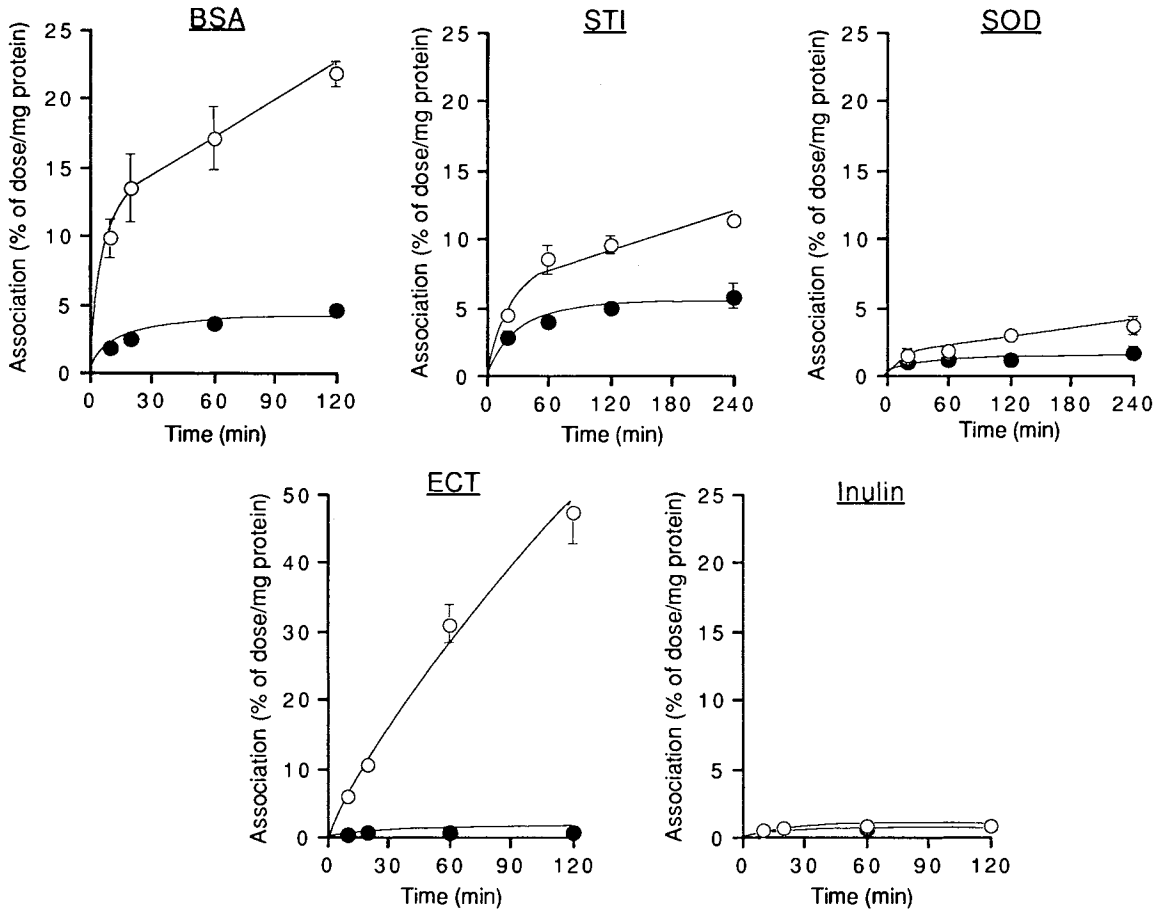

Fig. 1. Association time courses of ^{111}In-labeled protein drugs and [^{14}C]inulin at 37 °C (*open circles*) and 4 °C (*closed circles*) with LLC-PK$_1$ cells. LLC-PK$_1$ cells were incubated with HBSS (pH 7.4) containing [^{111}In]STI, [^{111}In]SOD, [^{111}In]BSA, and [^{111}In]Asu-ECT at the concentration of 1.3 μg/ml. Each value is expressed as the mean ± S.D. of 3 determinations. S.D. was included in the symbol when it was small.

ble to the rate of fluid-phase endocytosis (4.6 μl medium/mg protein/60 min) determined with [^{14}C]sucrose in LLC-PK$_1$ cells (9). In contrast to the minimal association of [^{14}C]inulin, which showed insignificant *in vivo* tubular reabsorption, significant and varied protein drug association profiles were observed (Fig. 1). In these experiments, we determined only the apparent cellular association of protein drugs that would

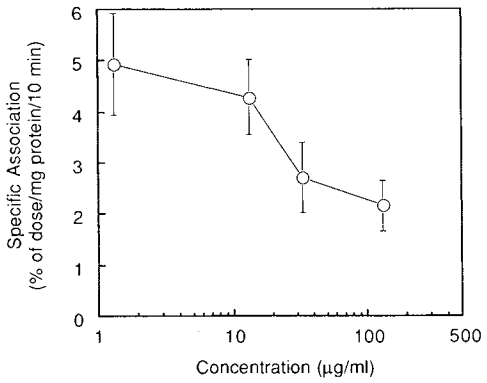

Fig. 2. Concentration dependence of [^{111}In]STI association with LLC-PK$_1$ cells. LLC-PK$_1$ cells were incubated in HBSS (pH 7.4) containing various concentrations of [^{111}In]STI for 10 min at 37 °C. Specific association was calculated by subtracting [^{14}C]inulin association at the same concentration from the total [^{111}In]STI association. Each value is expressed as the mean ± S.D. of 3 determinations.

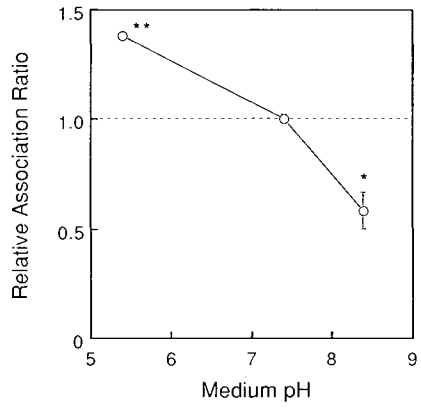

Fig. 3. Effects of medium pH on [^{111}In]STI association (2 hr, 37 °C) with LLC-PK$_1$ cells. Cellular association experiments were performed at pH 5.4, 7.4, and 8.4 and the association was expressed as the ratio to that at pH 7.4 (16.0 ± 0.4 % of dose/mg protein). Each value represents the mean ± S.D. of 3 determinations. *$p < 0.01$, **$p < 0.001$, significantly different from the control value by Student's *t*-test. S.D. was included in the symbol when it was small.

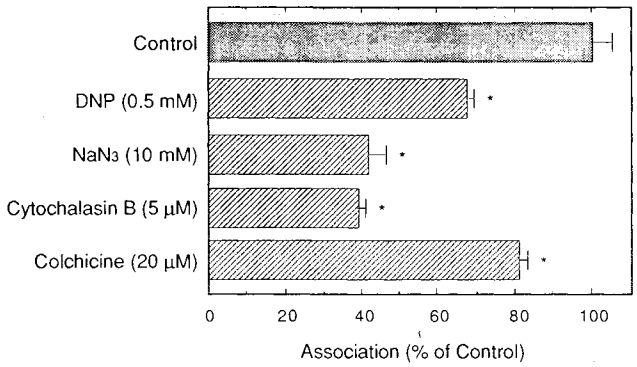

Fig. 4. Effects of metabolic and endocytosis inhibitors on [^{111}In]STI association with LLC-PK$_1$ cells. LLC-PK$_1$ cells were incubated in HBSS (pH 7.4) containing [^{111}In]STI (1.3 μg/ml) in the presence of metabolic and endocytosis inhibitors for 4 hr at 37 °C. Each value is expressed as the mean ± S.D. of 3 determinations. *$p < 0.001$, significantly different from the control value (17.6 ± 1.0 % of dose/mg protein) by the Student's t-test.

involve both adsorption on the cell surface and internalization. Although the inhibitory effects of metabolic and endocytosis inhibitors on the cellular association (Fig. 4) suggested that the proteins could be internalized by LLC-PK$_1$ cells, further studies, such as acid-wash experiments and detailed examinations with confocal laser microscopy, should be carried out to confirm such a phenomenon.

In reabsorption in the proximal tubule, proteins are, generally, first bound to the luminal cell surface, endocytosed, digested in the cell lysosomes, and then finally returned to the central circulation as amino acids (1,2). The reabsorption is a process of adsorptive endocytosis that is dependent on cellular energy; it is a saturable process of relatively high capacity and low affinity. It has been postulated that the binding process, the first step of the adsorptive endocytosis, is determined, in part, by an interaction between the positive charges of the protein molecule, such as those borne by the amino groups of lysine residues, and the negative surface charges of the renal epithelial cells (1,2). Although the molecular mechanism underlying the adsorptive endocytosis remains controversial (2,11), it does appear that the molecular charge and other properties of the protein, such as size and molecular configuration, are important factors.

The net charge of the protein seems to be one important determinant of the binding selectivity (12). Of the protein drugs tested in this study, we found that Asu-ECT, a basic polypeptide hormone, was most effectively associated with LLC-PK$_1$ cells, and we observed a marked temperature-dependence (Fig. 1). In addition, [^{111}In]Asu-ECT binding was not inhibited by other protein drugs that had net negative charge under the experimental conditions (pH 7.4). These results supported an important role for protein net charge in the binding of protein to the apical cellular membrane. Furthermore, it is conceivable that receptor-mediated binding may be involved in the effective association of the calcitonin analog, Asu-ECT, since LLC-PK$_1$ cells express functional calcitonin receptors (13).

It is generally accepted that proteins and large peptides are taken up by the tubular cells as intact molecules. In contrast, small linear peptides, such as angiotensin II and bradykinin (composed of 8 and 9 amino acids, respectively), are known to be degraded at the luminal surface of the brush border of the proximal tubules, which region contains many hydrolytic enzymes, by the process of membrane digestion with reabsorption of the breakdown products (2). In this study, involvement of this phenomenon in protein drug association is unlikely, since even the smallest protein we tested, Asu-ECT, is composed of 32 amino acids and contains an intramolecular bridge in its structure. No degradation products were detected in the incubation medium after the cellular association experiments.

The pH-dependent association of STI with LLC-PK$_1$ cells (Fig. 3) was similar to the observations of Christensen and Bjerke (14), who, using a micropuncture technique, showed that BSA uptake in the rat proximal tubule was significantly higher at pH 4.5 and 6.0 than at pH 7.4. The net charge of proteins with pI values of about 5 becomes more positive by reducing luminal pH. A more positive net charge of the protein will increase its affinity to the negative charges on the luminal surface of the brush border in the proximal tubule.

Mutual inhibition was observed in the competitive binding experiments with STI, SOD, and BSA, proteins with different molecular weights but that have net negative charge at pH 7.4 (Table II). Although further experiments to determine the affinity and capacity will be needed to discuss the binding specificity, these results suggested that they shared a common binding site on the luminal membrane of LLC-PK$_1$

Table II. Effect of Various Proteins on ^{111}In-labeled Protein Binding to LLC-PK$_1$ Cells

Proteins	Binding (% of Control)				
	Control	+STI	+SOD	+BSA	+ECT
[^{111}In]STI	100 ± 9.9	41.3 ± 8.4^c	46.8 ± 7.7^c	19.9 ± 7.0^d	50.6 ± 20.1^a
[^{111}In]SOD	100 ± 20.1	57.1 ± 9.3^a	80.7 ± 10.8	69.5 ± 5.8^a	57.2 ± 10.1^a
[^{111}In]BSA	100 ± 12.4	70.5 ± 6.5^a	63.4 ± 10.0^b	62.8 ± 7.4^b	n.d.
[^{111}In]Asu-ECT	100 ± 22.0	95.5 ± 5.6	n.d.	91.7 ± 2.9	38.1 ± 8.4^a

LLC-PK$_1$ cells were incubated with ^{111}In-labeled proteins (1 μg/well) in the presence of unlabeled another protein (100 μg/well) for 4 hours at 4 °C.

Control values were 3.8 ± 0.4, 1.7 ± 0.3, 4.5 ± 0.6, and 4.8 ± 1.4 (% of dose/mg protein) for STI, SOD, BSA, and Asu-ECT, respectively. Each data represents the mean ± S.D. of 3 determinations.

Significantly different from the control value by the Student's t-test ($^a p < 0.05$, $^b p < 0.02$, $^c p < 0.01$, $^d p < 0.001$)

N.D.; not determined.

cells. These findings imply that a variety of protein drugs could bind to LLC-PK_1 cells in a non-specific manner.

SOD has been employed as a therapeutic agent for various diseases mediated by reactive oxygen species, including renal failure (5). The present study showed that SOD association with LLC-PK_1 cells can be controlled by chemical modification. These results corresponded well to our previous results in isolated perfused rat kidneys (4). Cat-SOD showed greatly increased association with the cells compared with native SOD probably due to enhanced electrostatic interaction by cationization. The therapeutic efficacy of Cat-SOD against acute ischemic renal failure in rats (5) may be ascribed, in part, to this effective association with the tubular cells. On the other hand, reduced cellular association was observed for Man-SOD, indicating that glycosylation leads to less interaction with the luminal membrane of LLC-PK_1 cells. This phenomenon could be explained by decreasing binding due to the blocking of the amino groups of the SOD molecule by sugar moieties. Similarly, Kowlulu *et al.* (15) reported that reabsorption of glycated albumin by the proximal tubule was significantly less effective than that of the unmodified counterpart.

In conclusion, we demonstrated that a variety of protein drugs bound to the monolayer of LLC-PK_1 renal epithelial cells in a non-specific manner. We assume that subsequent endocytosis of the bound proteins would occur, although further studies are required to confirm this. LLC-PK_1 renal epithelial cells could be a useful *in vitro* model for studying the mechanism that underlies proximal tubular protein reabsorption.

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