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Transcellular transport of 4-iodo-L-*meta*-tyrosine via system L across monolayers of kidney epithelial cell Line LLC-PK$_1$

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Abstract

The substance 4-[^{125}I]iodo-L-*meta*-tyrosin (4-[^{125}I]*m*Tyr) is a radioiodinated amino acid that exhibits high *in vivo* stability and rapid renal elimination *in vivo*. We investigated transport of 4-[^{125}I]*m*Tyr in LLC-PK$_1$ (porcine kidney epithelial cell line) monolayers grown on collagen-coated, micro-porous membrane filters. We found that 4-[^{125}I]*m*Tyr transport in LLC-PK$_1$ cells was carrier-mediated and sodium-independent, and that 4-[^{125}I]*m*Tyr transport was similar to that of L-Tyr and 3-iodo-α-methyl-L-tyrosine. The results of the inhibition experiments suggest that 4-[^{125}I]*m*Tyr transport is predominantly mediated by a L-type amino acid transporter 1–like porcine homologue (a component of system L) in both basolateral and apical membrane.

Keywords: Amino acid transport; System L; Epithelial cell line; LLC-PK$_1$; 4-Iodo-L-*meta*-tyrosine

1. Introduction

In a recent study, we found that 4-[^{125}I]iodo-L-*meta*-tyrosine (4-[^{125}I]*m*Tyr; Fig. 1), an artificial amino acid, exhibits high metabolic stability and similar biodistribution to other radiolabeled L-Tyr analogs [1]. Recent reports indicate that 4-[^{125}I]*m*Tyr shows promise as a new amino acid transport marker.

Christensen [2] reported that three different transport systems (designated A, ASC and L) mediate transport of neutral amino acids across the plasma membrane of mammalian cells. System A is Na^+-dependent and most readily transports neutral amino acids with short, polar, or linear side chains. Specific inhibitors of system A are 2-(methylamino)isobutyric acid (MeAIB) and 2-aminoisobutyric acid (AIB). System ASC is also Na^+-dependent but excludes MeAIB. System L is Na^+-independent, and 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (BCH) acts as a specific substrate. System L is essential for cellular nutrition and trans-cellular transport of neutral amino acids at blood-tissue barriers of epithelia.

LLC-PK$_1$, an epithelial cell line from a Hampshire pig kidney, has a hexose and phosphate transport system with characteristics similar to those observed in the proximal tubule [3-6]. Confluent monolayers of LLC-PK$_1$ cells retain several of the characteristics of differentiated renal proximal tubular cells. For example, LLC-PK$_1$ forms an oriented monolayer with tight junctions and absorbs electrolytes and some nutrients via the microvilli of the apical membrane [6]. LLC-PK$_1$ cells are polarized epithelial cells with apical and basolateral membrane domains [3-6]. Incidentally, several characteristics of LLC-PK$_1$ cells are comparable to those of tumor cell lines. It has been reported that LLC-PK$_1$ cells grow rapidly in monolayer cultures. They are stable through more than 300 serial passages, and there is no evidence that the cells undergo transformation [6]. We speculated that studies of transcellular transport of artificial amino acids could be an informative new application of the LLC-PK$_1$ system. The purpose of the present study was to clarify mechanisms of transcellular transport of 4-[^{125}I]*m*-Tyr by LLC-PK$_1$ cell monolayers grown on collagen-coated, microporous membrane filters [3] (Fig. 2).

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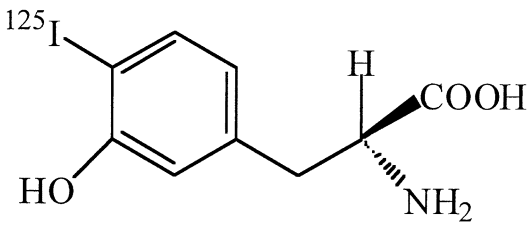

4-[^{125}I]iodo-L-*meta*-tyrosine (4-[^{125}I]*m*Tyr)

Fig. 1. Chemical structure of 4-[^{125}I]iodo-L-*meta*-tyrosine.

2. Methods and materials

2.1. Materials

D,L-*m*Tyr, BCH, MeAIB, AIB, and chloramine-T were acquired from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Natural L-amino acids and other chemicals of reagent grade were purchased from Kanto Chemical Co. (Tokyo, Japan). [^{125}I]NaI was obtained from Amersham Pharmacia Biotech UK (Buckinghamshire, UK). A Crownpak CR(−), a chiral column, (4 × 150 mm; Daicel Chemical Industries, Tokyo, Japan) and a Nova-Pak C18 (3.9 × 300 mm; Waters, Milford, MA) were used for separation and purification. A silica gel, thin-layer chromatography kit (TLC, catalogue number Art. 5553) was obtained from Merck (Darmstadt, Germany).

For cell line studies, LLC-PK$_1$ cells were obtained from Dainippon Pharmaceuticutical Co. (Osaka, Japan). Plastic tissue culture dishes (diameter 60 mm; Falcon; Becton Dickinson, Lincoln Park, NJ), plastic culture flasks (surface area, 25 cm^2; Nalge Nunc International, Roskilde, Denmark) and Transwell cell chambers (surface area, 4.71 cm^2; Costar, Cambridge, MA) were purchased.

2.2. Preparation of labeled compounds

Preparation of 4-[^{125}I]*m*Tyr was performed using a previously reported method [7,8]. Briefly, the racemic body of *m*Tyr was separated by HPLC using Crownpak CR (−). Chloramine-T (10 μL at 1.0 mmol/L in 0.05 mol/L phosphate buffer [pH 8.5]) was added to a mixture of L-*m*Tyr (100 μL at 1.0 mmol/L) and was carrier free [^{125}I]NaI (3.7-7.4 MBq) in 35 μL of 0.4 mol/L phosphate buffer (pH 8.5). The resulting solution was allowed to stand for 30 minutes, and 10 μL of 1.0 mmol/L sodium metabisulfite in 0.05 mol/L phosphate buffer (pH 8.5) was added. Geometric isomer separation by HPLC was then performed using a Nova-Pak C18 (eluent, 0.02 mol/L potassium acetate: ethanol [90:1]; flow rate, 0.5 mL/min; retention time for 4-isomer, 17-20 min; retention time for I$^-$, 4-5 min; retention time for cold mTyr, 5-7 min) [8]. Labeling efficiency and radiochemical purity were studied using silica gel TLC with two solvent systems: 1) methanol:acetic acid (100:1) (Rf value: 4-[^{125}I]*m*Tyr, 0.50; I$^-$, 0.80); and 2) methanol:10% ammonium acetate (10:1) (Rf value: 4-[^{125}I]*m*Tyr, 0.55; I$^-$, 0.80).

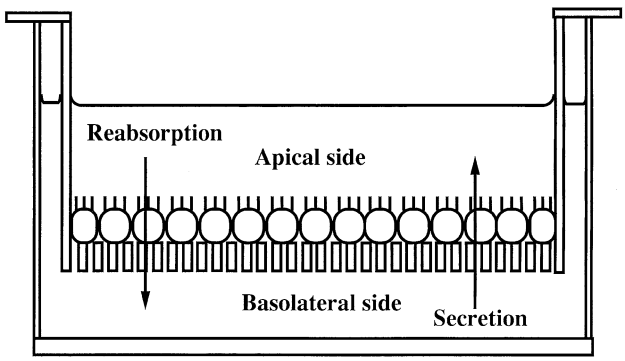

Fig. 2. LLC-PK$_1$ cell monolayer grown on a permeable support in Transwell cell chambers. It provides a cell culture model that exhibits several characteristics of in vivo epithelial membranes.

2.3. Cell cultures

Cell line studies were performed using modified methods described by Saitoh et al. [9], as follows. LLC-PK$_1$ cells were maintained by serial passages in 25-cm^2 cell culture flasks. The cells were fed with Dulbecco's modified Eagle's medium (Sigma-Aldrich Japan K.K., Tokyo, Japan) supplemented with L-glutamine (2 mmol/L) and 10% fetal bovine serum without antibiotics, in an atmosphere of 7.5% CO_2 and 95% air at 37°C (pH 7.4). Subculturing was performed every 5 days using 0.02% EDTA and 0.05% trypsin. Cells were used between passages 230 and 240.

2.4. Monolayer preparation

To prepare monolayers on a micropores support, cells were seeded at a density of 5 × 10^5 cells/cm^2 on polycarbonate membrane filter (pores, 3 μm) in Transwell cell chambers. The volume of medium inside and outside the Transwell chamber was 1.5 and 2.6 mL, respectively. Fresh medium was replaced every 2 days. The cells were used between days 4 and 5.

2.5. Measurement of transepithelial transport and cellular accumulation

Transepithelial transport and accumulation of 4-[^{125}I]*m*-Tyr were measured using monolayer cultures grown in the Transwell chambers. The incubation medium was Dulbecco's phosphate-buffered saline (PBS; pH 7.4) containing 137 mmol/L NaCl, 3 mmol/L KCl, 8 mmol/L Na_2HPO_2, 1 mmol/L $CaCl_2$, and 0.5 mmol/L MgCl. The pH of medium was adjusted by adding HCl or NaOH solution. In Na^+-free medium, the NaCl and Na_2HPO_4 normally present in PBS were replaced with choline chloride and K_2HPO_4, respectively. In general experiments, after the removal of culture medium from both sides of the monolayers, the cell mono-

layers were preincubated with 2 mL of incubation medium on each side for 10 minutes at 37°C. Then, 2 mL of incubation medium containing 4-[^{125}I]*m*Tyr (18.5 kBq) was added either to the basolateral or apical side, and 2 mL of radioactive free incubation medium were added to the opposite side. The monolayers were incubated for a specified period of time at 37°C or 4°C. [^{14}C]Inulin (18.5 kBq), a compound that is not transported by the cells, was used to evaluate paracellular fluxes and extracellular trapping of radioactivity. For inhibition studies, Tyr, 3-iodo-α-methyl-L-tyrosine (IMT), BCH, AIB, MeAIB, or Arg was added at a final concentration of 1 mmol/L. The cells were incubated for 90 minutes at 37°C with 18.5 kBq 4-[^{125}I]*m*Tyr. For the pH dependence experiment, the pH (hypothetic urine pH) of apical side incubation medium was adjusted to 5.0, 6.0, 7.0, or 8.0 by adding HCl or NaOH solution. The pH of the basolateral side was fixed at 7.4 (hypothetic blood pH). The cells were incubated for 90 minutes at 37°C with 18.5 kBq 4-[^{125}I]*m*Tyr added to apical or basolateral side.

For transport measurements, an aliquot (50 μL) of the incubation medium on the other side was taken at the specified time, and the radioactivity was counted. For accumulation studies, the medium was removed by suction at the end of the incubation period, and the monolayers were rapidly washed twice with 2 mL of ice-cold incubation medium on each side. The cells on the filters were solubilized in 0.5 mL of 1 N NaOH, and the radioactivity of each aliquot (100 μL) was counted. The ^{125}I and ^{14}C radioactivity of the collected media and the solubilized cell monolayers was determined using an ARC-1000M well-type scintillation counter (Aloka, Tokyo, Japan), and an LS6500 liquid scintillation counter (Beckman Instruments, Fullerton, CA) in Clear-sol II (Nacalai Tesque Inc., Kyoto, Japan).

Inhibition experiments were also performed using natural amino acids as inhibitors. LLC-PK$_1$ cells were seeded on 60-mm diameter plastic tissue culture dishes at a cell density of 5×10^5 cells/dish in 5 mL of complete medium, and were used on days 6 and 7 after inoculation. After removal of culture medium, each dish was washed once with 5 mL of incubation medium for 10 minutes at 37°C. Uptake was measured in the 2 mL of Na^+-free incubation medium. One of the natural amino acids was added to a final concentration of 1.0 mmol/L. Cells were incubated for 10 minutes at 37°C with 18.5 kBq of 4-[^{125}I]*m*Tyr. Thereafter, media were aspirated, and monolayers were rapidly rinsed twice using 5 mL of ice-cold incubation medium. Cells were solubilized in 1.5 mL of 1 N NaOH, and the radioactivity of each aliquot was counted. Radioactivity associated with solubilized cells was determined using an ARC-1000M well-type scintillation counter (Aloka).

2.6. Statistical analysis

The values obtained in each experiment were expressed as the mean ± SD. Statistical comparisons between groups were performed using the Student *t* test.

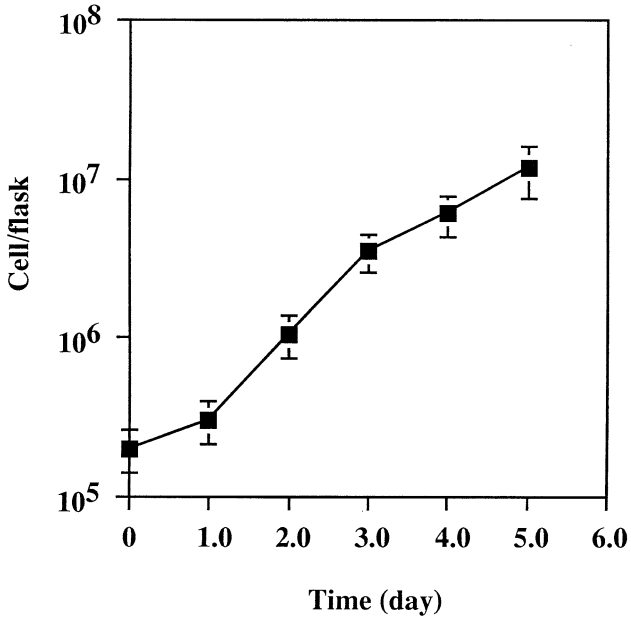

Fig. 3. Growth curve of LLC-PK$_1$.

3. Results

The labeling efficiency of 4-[^{125}I]*m*Tyr was greater than 80%. After purification, no-carrier-added 4-[^{125}I]*m*Tyr with radiochemical purities greater than 95% was obtained, and specific radioactivity was greater than 8.1×10^{19} Bq/mol.

Figure 3 shows the growth curve of LLC-PK$_1$. It took 4 days to reach confluence. Dome structure attributed to reabsorption of water appeared 4 days after inoculation (Fig. 4).

Figure 5A shows the transcellular transport of 4-[^{125}I]*m*-Tyr from the basolateral to apical side, and from the apical to basolateral side. Apical-to-basolateral transport of 4-[^{125}I]*m*Tyr (26.66 ± 2.82%) was much greater than basolateral-to-apical transport (11.65 ± 0.54%) for up to 120 minutes. Incubation at 4°C significantly decreased transcellular transport and accumulation of 4-[^{125}I]*m*Tyr ($P < 0.001$). Accumulation of 4-[^{125}I]*m*Tyr from the basolateral side (1.30 ± 0.08%) and apical side (2.19 ± 0.02%) was observed at 90 minutes of incubation (Fig. 5B). Apical-to-

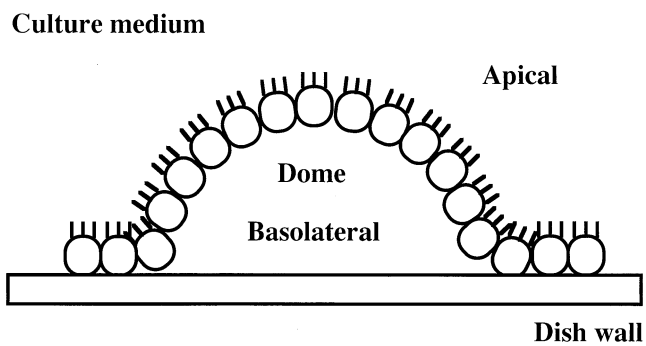

Fig. 4. Dome structure of LLC-PK$_1$ cell monolayers attributed to reabsorption of water.

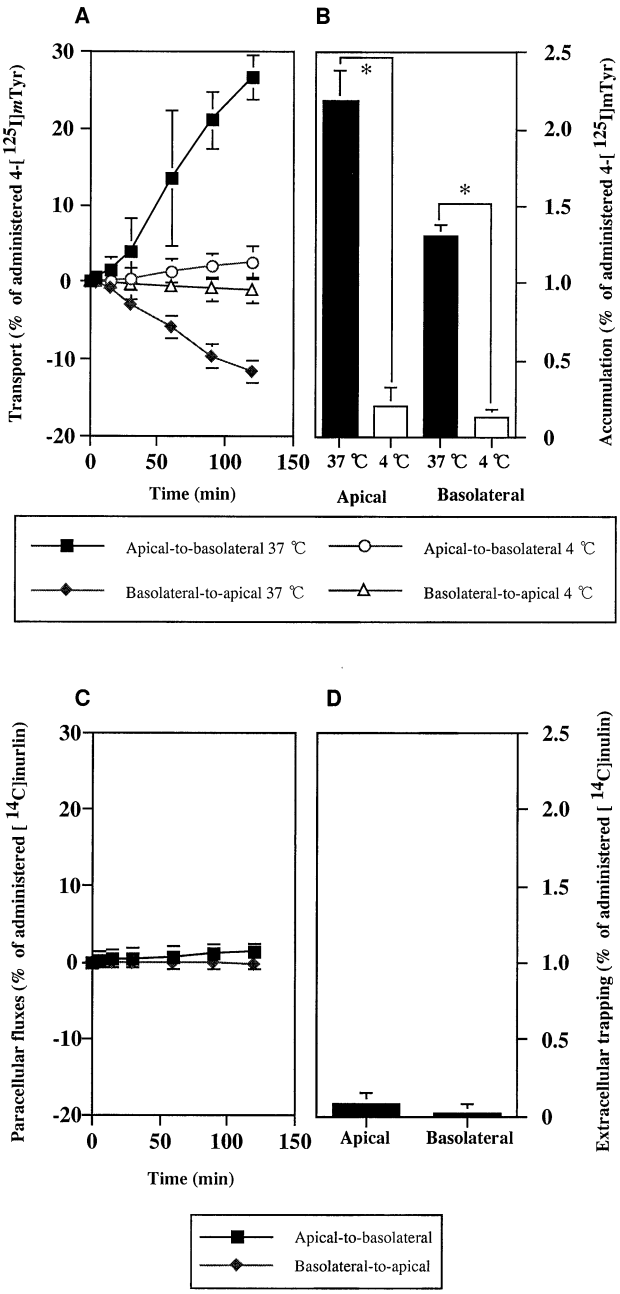

Fig. 5. Transcellular transport of 4-[^{125}I]*m*Tyr (A) and accumulation at 90 minutes of incubation (B), paracellular fluxes (C) and the extracellular trapping of [^{14}C]inulin (D). Values represent the mean ± SD (n = 4-5 monolayers). *$P < 0.001$.

basolateral and basolateral-to-apical leak of [^{14}C]inulin was $< 0.26\%$ and $< 0.07\%$ of the total [^{14}C]inulin, respectively, up to 120 minutes (Fig. 5C). The amount of [^{14}C]inulin trapped in the extracellular space from both sides was $<$ 0.003% of the total [^{14}C]inulin (Fig. 5D).

To examine substrate specificity of the basolateral and apical amino acid transport system in LLC-PK$_1$ cells, we evaluated effects of several amino acids (added to the basolateral or apical side) on transcellular transport and accumulation of 4-[^{125}I]*m*Tyr from the basolateral or apical side of the monolayers.

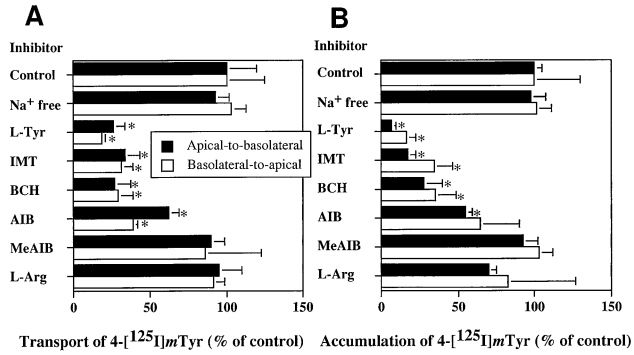

Fig. 6. Effects of inhibitors (1 mmol/L) on transcellular transport (A) and accumulation (B) of 4-[^{125}I]*m*Tyr. Each column represents mean ± SD of 3-5 LLC-PK$_1$ cell monolayers grown on a permeable support. *$P < 0.01$.

As shown in Fig. 6, at 1 mmol/L, the amino acids BCH, Tyr, and AIB inhibited accumulation ($P < 0.01$) as well as both basolateral-to-apical and apical-to-basolateral transport ($P < 0.01$) of 4-[^{125}I]*m*Tyr. IMT also inhibited 4-[^{125}I]*m*Tyr transport and accumulation from both sides ($P < 0.01$). MeAIB did not exhibit a significant inhibitory effect. Na^+-dependent transport did not significantly contribute to the bidirectional transport.

We evaluated effects of apical-side pH on transcellular transport and accumulation of 4-[^{125}I]*m*Tyr. As shown in Fig. 7, when pH of apical incubation buffer was varied from 5.0 to 8.0 (pH of basolateral side fixed at 7.4), accumulation of 4-[^{125}I]*m*Tyr from both basolateral and apical side was significantly affected ($P < 0.001$), whereas basolateral-to-apical and apical-to-basolateral transport were not significantly affected.

As shown in Fig. 8, uptake of 4-[^{125}I]*m*Tyr decreased (<30%) in the presence of neutral L-amino acids with large,

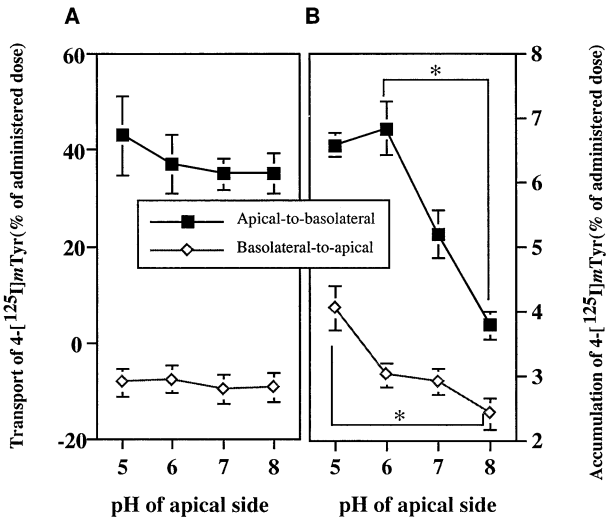

Fig. 7. Transcellular transport (A) and accumulation (B) of 4-[^{125}I]*m*Tyr depending on apical pH. *$P < 0.001$.

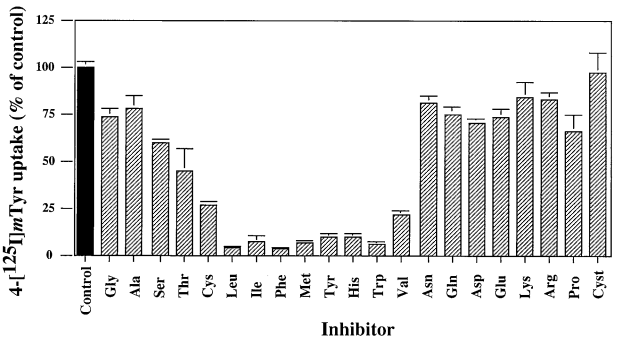

Fig. 8. The effects of natural L-amino acids (1 mmol/L) on 4-[^{125}I]*m*Tyr transport in apical membrane of LLC-PK$_1$ cell monolayers on dishes.

branched, or aromatic neutral side chains (i.e., Cys, Leu, Ile, Phe, Met, Tyr, His, Trp, and Val).

4. Discussion

As shown in Fig. 5, apparent bidirectional transcellular transport and accumulation of 4-[^{125}I]*m*Tyr were observed in the LLC-PK$_1$ cell monolayers. Lowered temperature significantly inhibited transport and accumulation, indicating that transport of 4-[^{125}I]*m*Tyr is due to carrier-mediated transport. Little of the administered dose of [^{14}C]inulin leaked to the opposite side of the LLC-PK$_1$monolayer. We attribute this and appearance of a dome structure on dishes 4 days after inoculation to formation of tight junctions in LLC-PK$_1$monolayers (Fig. 4).

In intact epithelial cells in the proximal tubule, basolateral membrane and apical membrane have different roles. Basolateral membrane is involved in transport between blood and tissue, and apical membrane is involved in transport between body and external environment. It is believed that these functions are related to the finding that basolateral membrane transport involves the L, A, and ASC systems, whereas in the apical membrane the b^{0+}, B^{0+}, B^0, A, and ASC systems contribute to neutral amino acid transport. In apical membrane of proximal tubule cells, these transport systems are predominantly involved in reabsorption of neutral and basic amino acids, including aromatic amino acids. System b^{0+} is a Na^+-independent transport system involved in transport of neutral amino acids (Leu, Ile, Val, Phe, Tyr, Trp, His, Met, Gln, Asn Thr, Cys, Ser, and Ala) and basic amino acids (Arg, Lys and Orn) [10]. System B^0 is a neutral amino acid transport system predominantly involved in reabsorption of neutral amino acids on the apical side of proximal tubule cells [11,12]. Palacin*et al* have suggested the presence of a Na^+-dependent transport system (System $B^{0,+}$) on the apical side of proximal tubule cells [13].

Mammalian L-type amino acid transporter 1 (LAT1) and L-type amino acid transporter 2 (LAT2), which are components of system L, are typical transporters for their respective roles. LAT1 is a major route for providing cells with large neutral amino acids [14,15]. The finding that LAT1 is conspicuously up-regulated in many kinds of tumor and transformed cell line is consistent with the observed increase in uptake of amino acids for rapid cell growth and proliferation [14]. LAT2 is responsible for neutral amino acid transport in basolateral membrane of proximal tubule cells, and for transferring smaller amino acids such as Ala, Ser, and Thr as well as large, branched, or aromatic neutral amino acids [15]. LAT1 and LAT2 require heavy chain of 4F2 cell surface antigen (4F2hc), a chaperone-like protein, for functional expression [14,15].

Uptake of [^{14}C]Leu via LAT1 is inhibited by Cys, Leu, Ile, Phe, Met, Tyr, His, Trp, and Val. Ala, Ser, and Thr have weaker inhibitory effects on LAT1-mediated [^{14}C]Leu uptake [14]. In contrast, Ala, Ser, Thr, Cys, Leu, Ile, Phe, Met, Tyr, His, Trp, Val, Asn, and Gln strongly inhibit LAT2-mediated [^{14}C]Leu uptake [15]. Data reported by Sepulveda et al. show that Ala and Ser weakly inhibit Leu uptake [5], suggesting that Na^+-independent uptake of neutral amino acids by LLC-PK$_1$ cells preferentially targets large and branched neutral amino acids as substrates (LAT1 substrates rather than LAT2 substrates). In the present inhibition experiment, transport of 4-[^{125}I]*m*Tyr was inhibited by BCH, L-Tyr and AIB, which are substrates of system L (Fig. 6). Furthermore, uptake of 4-[^{125}I]*m*Tyr decreased in the presence of large, branched, or aromatic neutral amino acids (Fig. 8). This substrate selectivity is very similar to reported selectivity of human and rat LAT1 [14,16]. LAT2 with 4F2hc is the prominent system L transporter in the basolateral membrane of intact proximal cells. However, the present results do not clarify the functional expression of LAT2, because although 4-[^{125}I]*m*Tyr clearly has an affinity to LAT1, it is unclear whether 4-[^{125}I]*m*Tyr has an affinity to LAT2. On the other hand, the similarity in inhibition between bidirectional transport and accumulation of 4-[^{125}I]*m*Tyr (Fig. 6) and the inhibition profiles of natural amino acids (Fig. 8) suggest that LAT1 with 4F2hc is present on both sides of LLC-PK$_1$ cell monolayers. This arrangement differs from that of intact epithelial cells in proximal tubule and is consistent with a previous report of system L-mediated transport of L-dopa, a neutral amino acid, in LLC-PK$_1$ cells [17]. It also indicates that the same carrier transports 4-[^{125}I]*m*Tyr, L-Tyr, and IMT (Fig. 6).

Lowered pH enhances uptake of Leu via system L [18]. As shown in Fig. 7, transcellular transport and accumulation from both directions were enhanced by lowered pH ($P < 0.001$). These observations show that transport and accumulation of 4-[^{125}I]*m*Tyr across the apical membrane can be stimulated by acidifying the medium on the apical side; that is, an inwardly directed H^+ gradient acts as a driving force for extrusion. This property also supports very high expression of system L–like transporter in LLC-PK$_1$ cells.

Transport of neutral amino acids by LLC-PK$_1$ cells is dominated by Na^+-independent transport, most likely involving LAT1, as indicated by the inhibitory effect of BCH under Na^+-free conditions. Studies of transport systems in

the apical membrane of LLC-PK_1 cells have shown the presence of system A and ASC [3,19], and the absence of Na^+-dependent neutral amino acid transport systems (B^{0+} and/or B^0) of the type responsible for Leu reabsorption by the proximal tubule *in vivo* [3,4,19]. In the present study, we observed very minor uptake (not significant) of 4-[^{125}I]*m*-Tyr into LLC-PK_1 cells via Na^+-dependent transport systems (Fig. 6). A lack of affinity of 4-[^{125}I]*m*Tyr for system A is suggested by the insignificant inhibitory effect of MeAIB (Fig. 6). AIB, a substrate of system ASC, had a significant inhibitory effect (Fig. 6). The results shown in Fig. 6 indicate that 4-[^{125}I]*m*Tyr transport and accumulation via Na^+-independent transport systems are inhibited by AIB. Expression of system ASC could not be excluded [3,4,19], but all transport of 4-[^{125}I]*m*Tyr was Na^+-independent. This suggests that 4-[^{125}I]*m*Tyr does not have an affinity with system ASC.

Several cell lines have been proposed as alternatives to LLC-PK_1, including MDCK and OK [5]. Characteristics of kidney epithelial cell lines depend on the renal tissue from which they are derived. These cell lines usually have different characteristics from intact cells of those tissues (e.g., up-regulation and defects of transporters), and these differences may affect results. With appropriate cell lines, the present experimental system is useful for studying transport of radiopharmaceuticals.

In conclusion, we investigated transcellular transport of 4-[^{125}I]*m*Tyr in LLC-PK_1 (porcine kidney epithelial cell line) monolayers grown on collagen-coated microporous membrane filters. We observed bidirectional transcellular transport of 4-[^{125}I]*m*Tyr by LLC-PK_1 cell monolayers. The system that transported 4-[^{125}I]*m*Tyr also transported L-Tyr and IMT. Na^+-dependent 4-[^{125}I]*m*Tyr uptake was insignificant. Transport of 4-[^{125}I]*m*Tyr by LLC-PK_1 cells is dominated by system L–like Na^+-independent transport (most likely via a porcine homologue of LAT1).

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