Urate Transport via Paracellular Route across Epithelial Cells

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INTRODUCTION

Urate is the final oxidation product of purine metabolism in humans. We have recently reported that the paracellular route is the major urate transport pathway across the blood–placental barrier. In this study, the mechanism of urate paracellular transport was investigated in several epithelial cell lines including Madin–Darby canine kidney (MDCK) type I, Lilly Laboratories cell-porcine kidney 1 (LLC-PK1) and Caco-2 cells. Very little urate passed through MDCK and LLC-PK1 cell layers. In contrast, one of the Caco-2 cell lines was found to be urate-permeable. This urate paracellular movement across Caco-2 cell layer was not inhibited by the urate transporter inhibitor benzbromarone but was partially inhibited by 4,4′-diisothiocyanato-2,2′-stilbenedisulfonic acid (DIDS), which inhibits chloride transport. Detection and quantification of claudin proteins that are important for paracellular transport of ions were performed by LC/MS. Claudins 1, 3, 4, 6, 7 and 12 were detected in urate-permeable cell lines, BeWo cells and Caco-2 cells. We compared claudin expression patterns in urate-permeable and urate-non-permeable Caco-2 cells by LC/MS and found that claudin 12 had a higher expression level in urate-permeable Caco-2 cells. Over-expression of these claudins in MDCK cells did not increase urate paracellular transport. Although there were differences in claudin expression patterns between urate-permeable and non-permeable cells, increased expression of single claudin alone did not explain paracellular permeability of urate.

Key words urate; epithelial cell; tight junction; paracellular transport; claudin

MATERIALS AND METHODS

Chemicals Radioactive [8–14C]-uric acid was purchased from American Radiolabeled Chemicals Inc. (Saint Louis, MO, U.S.A.). Fluorescein-conjugated dextran (molecular weight (MW), 3000) was obtained from Life Technologies (Carlsbad, CA, U.S.A.). 4,4′-Diisothiocyanato-2,2′-stilbenedisulfonic acid (DIDS) and benzbromarone were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Plasmid Construction Claudin constructs were cloned by remain elusive.

Claudins are tetraspanning membrane proteins and are expressed at tight junctions. Their expression pattern is critical for the barrier properties between cell–cell contact together with other junctional proteins, occludin and tricellulin. Twenty-seven mammalian claudin genes have been identified and each claudin has been shown to have different ion selectivity and barrier function. In example, result of experiments using knockout mice have indicated that claudins 1, 5, 11 and 14 form a cationic barrier in many tissues such as the skin, blood brain barrier and Sertoli cells.

In contrast, claudins 2, 7, 10, 15 and 16 have been identified as cationic ion-selective pores. Since urate exists in an anionic form in physiological fluid, we hypothesized that specific anion-permeable claudins are responsible for paracellular urate transport.

In this study, urate paracellular transport and claudin expression were investigated in several epithelial cell lines.
PCR was routinely performed with KOD DNA polymerase (Takara Bio Inc., Shiga, Japan) for 30 cycles with 95°C denature for 10s, 55°C annealing for 15s and 68°C extension for 1 min according to the product manual. PCR was carried out with primers that included HindIII, EcoRI or MfeI restriction sites and unrelated six bases. The flag tag was fused to the carboxyl termini of claudins. The PCR fragments were subcloned into the mammalian expression vector pcDNA 3.1 (+), which already has the flag epitope tag sequence. PCR primer sequences are shown in Supplementary Table S1.

Cell Culture and Transfection  CW-2 cells (human colon carcinoma cells) were purchased from RIKEN BioResource Center (Tsukuba, Japan). Lilly Laboratories cell-porcine kidney 1 (LLC-PK1) cells (pig kidney cells) and Madin–Darby canine kidney (MDCK) type I cells (dog kidney cells) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, U.S.A.). MDCK type II and Caco-2 cells were kind gifts from Dr. Takada (Tokyo University). All cell lines except for CW-2 cells were cultured in a humidified incubator under 5% CO2 in 100 U/mL penicillin, and 100 µg/mL streptomycin. CW-2 cells were a kind gift from Dr. Caplan (Yale University) and other Caco-2 cells were a kind gift from Dr. Takada (Tokyo University). All cell lines were grown to confluence on cell culture inserts (BD, Franklin Lakes, NJ, U.S.A.). MDCK type II and Caco-2 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, U.S.A.).

Preparation of a Membrane Fraction  Cells or minced tissues were washed with cold PBS and homogenized with a dounce-homogenizer in a buffer containing 0.5 mM MgCl2, 10 mM Tris–HCl, pH 7.4 and a protease inhibitor cocktail (Sigma-Aldrich). An equivalent volume of sucrose solution containing 0.5 M sucrose and 10 mM Tris–HCl, pH 7.4 was added and the mixture was homogenized again. The obtained homogenates were centrifuged at 10000 × g for 10 min at 4°C, and the supernatants were collected and ultracentrifuged at 100000 × g for 30 min at 4°C. The microsomal pellet was suspended in Tris–sucrose buffer containing 250 mM sucrose and 10 mM Tris–HCl, pH 7.4. The sample was layered on top of a 38% (w/v) sucrose solution and centrifuged at 100000 × g for 30 min at 4°C. The turbid layer at the interface was recovered, suspended in the Tris–sucrose buffer, and centrifuged at 100000 × g for 30 min at 4°C. The plasma membrane fraction was obtained from the resulting pellet, which was suspended in Tris–sucrose buffer. Protein concentration was measured using Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.) with bovine serum albumin as a standard.

In Solution Digestion and Tandem Mass Tag™ (TMT) Labeling for MS Analysis  The membrane fraction solubilized in 100 mM of ammonium bicarbonate solution containing 12 mM sodium deoxycholate and 12 mM sodium N-lauroyl sarcosinate was digested with trypsin (Proteomics grade; Roche Life Science, Switzerland). Tryptic digests were treated according to the Phase-transfer surfactants (PTS) protocol and desalted using C18-StageTips. Briefly, membrane proteins were reduced with 10 mM dithiothreitol for 30 min at 55°C, alkylated with 60 mM iodoacetamide for 30 min at room temperature, and digested with 1:20 (w/w) trypsin for 16 h at 37°C. An equal volume of an organic solvent, ethyl acetate, was added to the digested samples, and the mixtures were acidified by 1% trifluoroacetic acid and vortexed to transfer detergents to the organic phase. After centrifugation, the aqueous phase containing peptides was collected and desalted using C18-StageTips. The tryptic peptides were labeled with TMT Reagents (Thermo Fischer Scientific, Bremen, Germany) following the manufacturer’s instructions. The labeled peptide sample was fractionated by SDB-StageTips with reverse phase in basic pH and fractionates were cleaned up and concentrated with C18-StageTips.

Protein Identification and Quantification by NanoLCMS/MS with Reverse Phase  Each fractionated peptide prepared as described above was injected into a trap column (C18, 0.3 × 5 mm; L-column, Chemicals Evaluation and Research Institute, Tokyo, Japan) and an analytical column (C18, 0.075 × 120 mm; Nikkky Technos, Tokyo, Japan), which
was attached to a NanoLC-MS/MS system. NanoLC-MS/MS analysis was conducted by an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) equipped with a nanoLC interface (KYA, Tokyo, Japan) and a nanoHPLC system DiNa (KYA). Purified peptides were introduced from NanoLC to the LTQ Orbitrap Velos, a hybrid ion-trap Fourier transform mass spectrometer. Full MS and MS/MS scans were followed by higher energy collisionally activated dissociation (HCD). The database search engines Proteome Discoverer 1.4 (Thermo Scientific) and MASCOT 2.4 (Matrix Science) were used to identify and quantify proteins from MS, MS/MS, and reporter ion spectra of peptides. Peptide mass data were matched by searching the UniprotKB/Swiss-prot database. False discovery rate (FDR) was calculated by enabling peptide sequence analysis using Percolator. High confidence peptide identification was obtained by setting a target FDR threshold of ≤1.0% at the peptide level.

Quantification of Claudin Proteins Trypsin-digested samples were prepared as described previously. Briefly, the membrane fraction (50 µg) was suspended in 100 mM Tris–HCl (pH 8.5), 7.5 M guanidinium hydrochloride, and 10 mM ethylenediaminetetraacetic acid (EDTA). The samples were reduced with dithiothreitol at room temperature for 60 min and S-carbamoylmethylated by treatment with iodoacetamide at room temperature for 60 min. The alkylated proteins were precipitated with a mixture of methanol and chloroform. The precipitates were dissolved in 6 M urea and diluted with 100 mM Tris–HCl (pH 8.0). The proteins were digested by lysyl endopeptidase (WAKO) at an enzyme/substrate ratio of 1:100 at 37°C for 3 h and by trypsin (Promega) at an enzyme/substrate ratio of 1:100 at 37°C for 16 h. The digested peptides were desalted by GLGC and SDB tip (GL Science) and dissolved in 0.1% formic acid.

The digested peptides was injected into a trap column (C18, 0.1 × 20 mm; PepMap 100, Thermo Fisher Scientific) and an analytical column (C18, 0.075 × 250 mm; PepMap RSLC-column, Thermo Fisher Scientific), which was attached to a NanoLC-MS/MS system (Ultimate 3000, Thermo Fisher Scientific). NanoLC-MS/MS analysis was conducted by using TripleTOF5600 mass spectrometer (Sciex) with a flow rate of 300 nL/min. For protein identification, MS data were acquired by information-dependent acquisition and analyzed by ProteinPilot 4 (Sciex) with a UniProt human reference proteome database. The detected precursor ion, fragment ion and retention time of claudins were used in the following quantification analysis. For quantification, MS data were acquired by SWATH. Then fragment chromatograms were extracted from the MS data and the peaks of the target peptides were identified on the basis of protein identification information using Skyline (Washington University). Claudin-1 was quantified by 10 fragment ions from 2 specific peptides, claudin-7 was quantified by 10 fragment ions from 1 specific peptide, and claudin-12 was quantified by 6 fragment ions from 1 specific peptide. The peak area ratio of each fragment ion was calculated from average peak areas of 2 independent trypsin digestions. The protein expression ratio of each claudin was calculated from the peak area ratios of fragment ions.

Ethics Statement Placental tissues were obtained from patients undergoing cesarean sections at 26 to 41 weeks of gestation in Kyorin University Hospital. All patients gave informed consent for collection and investigational use of tissues. This study protocol was approved by the ethics committee of Kyorin University School of Medicine (permit number 378-04).

RESULTS

Formation of Functional Tight Junctions Epithelial cells form tight junctions and make electrical resistance between apical and basolateral membranes. To confirm the formation of tight junctions, cells were grown on cell culture inserts and transepithelial electrical resistance (TER) was measured (Table 1). TER of CW-2 cell monolayers, which do not form tight junctions (negative control), was around 8.0 Ω·cm². In contrast, MDCK type II and LLC-PK1 cell monolayers had much higher TER (around 50 Ω·cm²). TER of MDCK type I and Caco-2 cell monolayers was more than 900.0 Ω·cm². Permeability of fluorescence-conjugated dextran showed similar results: FITC-dextran leaked through CW-2 cell monolayers but not other monolayers (supplementary Fig. S1). These results indicate that MDCK and Caco-2 cells form functional tight junctions.

Paracellular Transport of Urate Paracellular urate transport was measured in both directions between the upper and lower chambers for 5 min and 15 min at 4°C, a temperature at which transporters should be inactive. As shown in Fig. 1A, very little urate passed through LLC-PK1, MDCK type I and MDCK type II cell layers in either direction. In contrast, significant urate movement was observed in one of the Caco-2 cell lines (A). No significant amount of urate was detected inside Caco-2 (A) cells during paracellular urate movement; the amount of urate inside cells was less than 1/50 by radioactive count compared with that of urate transported by paracellular route. Thus, it is unlikely that urate movement through Caco-2 (A) cell monolayers takes the transcellular route.

As expected, this urate paracellular movement of the Caco-2 cell monolayer at 4°C was hardly inhibited by urate transporter inhibitors including benz bromarone (Fig. 1B), fumitremorgin C and glibenclamide (data not shown). Interestingly, the urate paracellular movement was partially inhibited by a high concentration of DIDS, which is an inhibitor of chloride transport (Fig. 1B).

Expression of Claudins in Urate-Permeable Tissue and Cells Claudins are important molecules in paracellular transport for ions. Since urate exists as an organic anion form in physiological pH, we speculated that claudins may be responsible for urate paracellular transport. RNA expression levels of claudins between urate permeable and non-permeable Caco-2 cell lines by RT-PCR showed no significant differences (data not shown). Then, claudin protein expression levels in the membrane fractions of urate-permeable and urate-non-

| Cells         | TER (Ω·cm²) |
|---------------|-------------|
| CW-2          | 8.36 ± 1.72 |
| Caco-2 (A)    | 121 ± 29.0  |
| Caco-2 (B)    | 382 ± 86.9  |
| MDCK type I   | 912 ± 44.2  |
| MDCK type II  | 54.1 ± 1.85 |
| LLC-PK1       | 57.6 ± 1.65 |
represents the mean area ratios of fragment ions (6–10 ions) from the specific tryptic peptides. Each data subjected to LC/MS analysis and claudins 1, 3, 4, 6, 7 and 12
swell cell culture inserts to full confluency. [14C]-urate was added to upper or lower wells and incubated
for 5 or 15 min. After incubation, the medium in the opposite wells was collected and radio activities were measured. B. Caco-2 (A) cells were cultured on Tran-
Fig. 1. Paracellular Transport of Urate through Cell Monolayers
A. Cell lines were cultured on Transwell cell culture inserts to full confluency. Urate movement was measured at 4°C to avoid influence of transporters and endocytosis/exocytosis. [14C]-urate was added to upper or lower wells and incubated for 5 or 15 min. After incubation, the medium in the opposite wells was collected and radio activities were measured. Student’s t-test was used to compare experimental results. p-Value less than 0.01 was considered significant.

Table 2. Comparison of Claudin Expression in Caco-2 Cell Lines

| Claudin      | Ratio of expression |
|--------------|---------------------|
|             | Caco-2 (A)/Caco-2 (B) |
| Claudin 1    | 0.381 ± 0.061       |
| Claudin 7    | 1.34 ± 0.05         |
| Claudin 12   | 2.22 ± 0.11         |

The protein expression ratio of each claudin subtype was determined from peak area ratios of fragment ions (6–10 ions) from the specific tryptic peptides. Each data represents the mean ± standard error of the mean (S.E.M.).

peptides were detected. Tight junction and adherens junction proteins such as occludin, ZO-1 and cadherins were detected by LC/MS. Their expression levels were not significantly different (less than 1.5 times) among the cells and the tissue examined.

**Overexpression of Claudins and Urate Paracellular Transport**
To determine the effects of these claudins on urate paracellular transport, Caco-2 cells were transfected with claudins, and stably expressed cell lines were selected (Figs. 2A, B). Since claudin 1 and claudin 12 were differently expressed in Caco-2 (B) cells and Caco-2 (A) cells, the effect of each claudin was tested in Caco-2 cell lines. When claudin 1, which was more abundant in urate-non-permeable cells, was overexpressed in urate-permeable Caco-2 (A) cells (Fig. 2A), their urate permeability was not affected (Table 3). Similarly, when urate-non-permeable Caco-2 (B) cells were transfected with claudin 12, which was expressed more in urate permeable cells (Fig. 2B), paracellular urate transport did not become detectable (Table 3). Moreover, when claudins 1, 3, 4, 6, 7 and 12, which were detected in urate-permeable tissue and cells, were overexpressed in urate-non-permeable MDCK type II cells (Figs. 2C–H), paracellular transport in these cell lines did not increase from that in wild-type MDCK type II cells (Table 3).

**DISCUSSION**

The paracellular route is an important path as well as transcellular route for ion transport though the epithelial cell layer. In the case of urate, transcellular transport has been extensively studied since many urate transporters have been cloned and characterized. In addition, it has been reported that loss-of-function mutations in some of the urate transporters lead to hypouricemia and hyperuricemia.1,6,9) In contrast, there are little information about paracellular transport of urate. It has been reported that urate is transported by the paracellular route in avian renal proximal tubules based on the fact that there is no urate in the cells during urate movement.12) Ogura et al. showed by using Ussing-type diffusion chambers that urate passes through the rat intestine by the transcellular route as well as the paracellular route.13) We have also demonstrated that there was no significant difference between serum urate levels in maternal blood and umbilical cord blood, suggesting that urate is freely movable at the placenta and that urate moves in monolayers of the placental model cell line BeWo through the paracellular route by simple diffusion.13) However, the mechanism of urate paracellular transport has not been fully elucidated.

Benzbromarone is a uricosuric agent and is the strongest inhibitor of urate transporters. Almost all of the urate transporter including URAT1, NPT1, NPT4, OAT1, OAT3, OAT4, OAT10, GLUT9/URATv1, ABCG2, and MRP4 are inhibited by benzbromarone. Reasoning that such a specific inhibitor for urate binding site of many urate transporters might inhibit urate paracellular movement, we tested whether benzbromarone inhibits urate paracellular transport through a Caco-2 monolayer. Urate paracellular movement was not affected (Fig. 1B). Other inhibitors of urate transporters including the organic anion transporter inhibitor probenecid, the ABCG2 inhibitor furmitremorgin C and the MRP inhibitor glibenclamide, did not inhibit urate movement (data not shown).
Similar results were obtained when BeWo cells, trophoblast-derived cells, were used.11) These results indicate that urate binding site of urate transporters is not relevant to urate paracellular transport.

DIDS is often used as an inhibitor of anion channels or transporters. Since urate exists as an anion form at physiological pH, we tested the effect of DIDS on urate paracellular transport (Fig. 1B). A high concentration of DIDS partially inhibited urate paracellular transport at 4°C, where urate transporters should not work. DIDS contains benzenesulfonic acid in its structure, and its sulfo group is ionized with a negative charge in a solution. Since both urate and DIDS are organic anions, DIDS may pass through the ion pores same as urate and urate paracellular transport was partially inhibited by DIDS. It has been reported that organic anions such as oxalate, furosemide, sodium dodecyl sulfate and 5-aminosalicylate are also transported via the paracellular route.23–26)

Claudins are tight junction proteins and they are responsible for paracellular permeability and barrier function of ions. Seventeen claudin isoforms in humans have so far reported. Different kinds of claudins are expressed in different epithelial cells and they can make either an ion pore or ion barrier. We detected higher expression levels of claudin 12 and claudin 1 in urate-permeable Caco-2 cells (A) and urate-non-permeable Caco-2 cells (B), respectively. To our surprise, overexpression of claudin 1 in urate-permeable cells and overexpression of claudin 12 in urate-non-permeable cells had no effect on urate paracellular transport. Then we tried to detect all claudins expressed in urate-permeable Caco-2 cells and placenta tissue. Each detected claudin was transfected into urate-non-permeable MDCK II cells, but urate paracellular transport was not enhanced by overexpression of any of the claudins.

There are several possibilities for urate paracellular transport. One is that combination of several claudins determine the paracellular permeability of urate. Hou ea al. demonstrated that co-expression of claudin 16 and claudin 19 in cultured renal epithelial cells generates paracellular cation selectivity, whereas expression of either claudin alone does not.27) They also reported that claudin 16 staining completely disappeared from tight junctions of the thick ascending limb and that co-oligomerization of claudin 16 with claudin 19 is required for paracellular channel function in the thick ascending limb.27) It has also been reported that claudin 8 is essential for tight

### Table 3. Urate Permeability across Monolayers of Claudin Over-Expressed Cells

| Transfected claudin | Cell line   | Urate permeability |
|---------------------|-------------|--------------------|
| Claudin 1           | MDCK type II| No change          |
| Claudin 3           | MDCK type II| No change          |
| Claudin 4           | MDCK type II| No change          |
| Claudin 6           | MDCK type II| No change          |
| Claudin 7           | MDCK type II| No change          |
| Claudin 12          | MDCK type II| No change          |
| Claudin 1           | Caco-2 (A)  | No change          |
| Claudin 12          | Caco-2 (B)  | No change          |

Fig. 2. Localization of Claudins Overexpressed in Caco-2 Cells and MDCK Type II Cells

Indicated claudins were transfected into Caco-2 or MDCK cells. Localization of claudins were visualized with immunohistochemistry stained by anti-flag antibody and Alexa Fluor 488 conjugated secondary antibody.
junction localization of claudin 4 in mouse collecting duct cells and that knock down of either claudin 4 or claudin 8 by siRNA leads to down-regulation of chloride paracellular permeability due to claudin 4.38) We are planning to knock down claudins in our system in the future.

Another possibility is that expressed claudin(s) forms aqueous pores with different size,16,29,39) through which urate can move by a solvent drug. It is known that there are aqueous pores in an epithelial cell layer and that neutral hydrophilic solutes, protonated weak bases and weak organic anions pass through the pores by the paracellular route. Adson et al. calculated the pore size in a Caco-2 cell monolayer from the degrees of permeability and molecular radii of several chemicals.31) They demonstrated that small molecules such as methylamine, urea and acetate are transported well via the paracellular pathway. Knipp et al. reported similar results for a Caco-2 cell monolayer; however, the calculated pore size was different.32) They suggested that the difference may be attributed to the experimental conditions under which the cell monolayers were grown and/or the transport studies were conducted. The different culturing conditions such as collagen-coated versus non-collagen-coated filters, 21–28 d of culture versus 13–16 d of culture, 37°C versus 25°C incubation temperature could have resulted in different cell densities, which could result in different pore sizes (i.e., a lower cell density could result in a larger pore size, whereas a higher cell density could result in a smaller pore size). If urate passes through aqueous pores, the pore size in each cell layer would be important. Linnankoski et al. calculated the pore porosity and size by determining the transport of paracellular polyethylene glycols in MDCK type II, Caco-2 and 2/4/A1 cell lines and the human intestine.33) They showed that there are two distinct pore sizes in MDCK type II cells, Caco-2 cells and the human intestinal epithelium and that the radii of the small and large pores are 5.5–6.6 and 10.1–30.5 Å, respectively. The 2/4/A1 cell line has only one pore size and its radius is 14.9 Å. Van Italie et al. suggested that porcine ileum MDCK cell lines (with high TER and low TER) and a Caco-2 cell line have small pores with similar radii of about 4 Å and that the number of pores is quite variable among the cell lines according to the result of a polyethylene glycols permeability assay.30) They also demonstrated that overexpression of claudin 2, but not that of claudins 4, 14 or 18, in MDCK cells increased the number of pores without altering the pore size. Thus, cell lines and tissues form distinct size pores according to the expression profiles of the kinds of claudins, and the different pore sizes may affect the paracellular transport of small molecule solutes including urate. There is no information about the diameter of urate in a fluid, but it seems from the results of this study that urate anion is larger than the pore size made by a single claudin that is overexpressed. It is possible that the pores for water and a solvent that are larger than ions exist in urate-permeable cell lines. In such a case, partial inhibition of urate transport by anion DIDS can be explained at the urate pore. There is some evidence for claudin forming water pores; there has been reported that claudin 2 expression is required for paracellular water transport under iso-osmotic conditions in proximal tubular cells.34) However, if paracellular transport of urate is only determined by claudin water pore size and density (porosity), TER would have been correlated well with urate permeability. As shown in Table 1 and Fig. 1, they were not correlated and the fact that only high concentrations of anion DIDS partially inhibited urate transport suggests some anion selective pore is responsible for urate paracellular transport.

In conclusion, we have demonstrated that urate paracellular transport is partially inhibited by an organic anion, DIDS, and that single overexpression of claudins that are expressed in urate-permeable cell line has no effect on urate transport via the paracellular route.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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