Localization of broadly selective equilibrative and concentrative nucleoside transporters, hENT1 and hCNT3, in human kidney

Vijaya L. Damaraju,1,2,3,6 Adam N. Elwi,1,2,3,6 Charlene Hunter,4 Pat Carpenter,2,3 Cheryl Santos,2,3 Gerry M. Barron,2,3 Xuejun Sun,2,3 Stephen A. Baldwin,5 James D. Young,1,6 John R. Mackey,2,7 Michael B. Sawyer,2,7 and Carol E. Cass1,2,3,8

1Membrane Protein Research Group and the Departments of 4Biochemistry, 5Laboratory Medicine and Pathology, 2Oncology and 6Physiology, University of Alberta, Edmonton, Alberta; Departments of 3Experimental Oncology and 7Medical Oncology, Cross Cancer Institute, Edmonton, Alberta, Canada; and the 8Institute of Membrane and Systems Biology, University of Leeds, Leeds, United Kingdom

Submitted 4 January 2007; accepted in final form 27 March 2007

Damaraju VL, Elwi AN, Hunter C, Carpenter P, Santos C, Barron GM, Sun X, Baldwin SA, Young JD, Mackey JR, Sawyer M, Cass CE. Localization of broadly selective equilibrative and concentrative nucleoside transporters, hENT1 and hCNT3, in human kidney. Am J Physiol Renal Physiol 293:F200–F211, 2007. First published April 4, 2007; doi:10.1152/ajprenal.00007.2007.—Nucleoside transporters in kidney mediate renal reabsorption and secretion of nucleosides. Using RT-PCR, we demonstrated mRNAs encoding hENT1, hENT2, hCNT1, hCNT2, and hCNT3 in both cortex and medulla. Immunoblotting with crude membrane preparations revealed abundant hENT1 and hCNT3 in both cortex and medulla, and little, if any, hENT2, hCNT1, or hCNT2, indicating that the latter were either absent or below limits of detection of immunoreactivity. hENT1 immunostaining was observed on apical surfaces of proximal tubules and on both apical and basal surfaces of thick ascending loops of Henle and collecting ducts. Prominent hCNT3 immunostaining was observed on apical surfaces of proximal tubules and thick ascending loops of Henle in addition to some cytoplasmic staining. Equilibrium binding of \[^{3}H\]nitrobenzylmercaptopurine ribonucleoside (NBMPR), a high-affinity inhibitor of hENT1, to brush-border membrane vesicles from cortex confirmed the presence of hENT1 on apical surfaces of proximal tubules. Uptake of \[^{3}H\]uridine by polarized renal proximal tubule cells exhibited a sodium-dependent component that was inhibited by thymidine and inosine as well as a sodium-independent component that was partially inhibited by NBMPR and completely inhibited by dipyridamole, indicating high levels of hENT1 and hCNT3 and low levels of hENT2 activities. The presence of \(^1\) transcripts for hENT1/2 and hCNT1/2/3 and the hENT1 and hCNT3 proteins in human kidneys and \(^2\) hENT1, hENT2, and hCNT3 activities in cultured proximal tubule cells suggest involvement of hENT1, hCNT3, and possibly also hENT2 in renal handling of nucleosides and nucleoside drugs.

renal transport of nucleosides; tissue distribution of nucleoside transporters

UPTAKE AND RELEASE of physiological nucleosides and most nucleoside drugs in human (h) cells are dependent on the activity of one or more members of two families of nucleoside transporters, the equilibrative and concentrative nucleoside transporters (hENTs, hCNTs) (2, 7). Two of the four known hENTs have broad permeant selectivities and wide tissue distributions (17) and are functionally distinguished by their relative sensitivities to inhibition by nitrobenzylmercaptopurine ribonucleoside (NBMPR) as either highly sensitive (hENT1) or relatively insensitive (hENT2). Although both transporters are inhibited by dipyridamole, dilazep, and drafalazine, hENT1 is much more susceptible than hENT2. The other two hENTs (hENT3, hENT4) are more specialized; hENT3 is a broadly selective organellar transporter and hENT4 transports adenosine and monoamines (3). hCNTs are found primarily in specialized tissues, including intestine, kidney, liver, and choroid plexus (2). Three different transporters (hCNT1, hCNT2, hCNT3) have been identified by molecular cloning (41, 43, 44, 52). hCNT1, hCNT2, and hCNT3 mediate, respectively, pyrimidine nucleoside-selective, purine nucleoside-selective, and broadly selective nucleoside transport activities.

Pharmacokinetic evidence suggests that some nucleosides are actively reabsorbed and secreted by the kidney (23, 39). Adenosine, a regulatory nucleoside that acts through binding to purinergic receptors, is found in plasma and its reabsorption in human kidney has been demonstrated. Several nucleosides have been shown in studies with rodents to be secreted by kidney, and it has been suggested that toxic nucleosides (e.g., 2’-deoxyadenosine) are selectively eliminated by renal secretion (24, 38). Renal reabsorption and secretion appear to involve different transport systems, since renal reabsorption of adenosine in mice was unaffected by classical nucleoside transport inhibitors (e.g., NBMPR and dipyridamole), whereas renal secretion of 2’-deoxyadenosine was decreased by treatment with these inhibitors (39). There is also a growing body of evidence that organic cation transporters may be involved in renal secretion of nucleosides (9, 32, 39).

Numerous studies with isolated membranes and renal cell lines have demonstrated functional nucleoside transporters in mammalian kidneys or cells derived therefrom. Separate concentrative transport activities for purine and pyrimidine nucleosides were shown with brush-border membrane vesicles (BBMV) from rat kidney (28), and other studies with BBMV from renal cortex from rats (29–31), rabbits (55), and cows (56) also demonstrated concentrative sodium-dependent transport activities. NBMPR-binding sites were detected on rat kidney membranes (54) and a basolateral NBMPR-sensitive nucleoside transport activity was shown in rabbit basolateral membrane vesicles (55). The opossum kidney OK1 (12) and

*V. L. Damaraju and A. N. Elwi contributed equally to this study.

Address for reprint requests and other correspondence: C. E. Cass, Dept. of Oncology, Cross Cancer Institute, 11560 Univ. Ave., Edmonton, Alberta T6G 1Z2, Canada (e-mail: carol.cass@cancerboard.ab.ca).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
pig kidney LLC-PK1 (16) cell lines exhibit equilibrative transport activities and small components of concentrative transport activities.

Nucleoside transporters have been demonstrated in human kidney by both functional studies and molecular cloning. Functional studies with human kidney BBMV revealed a single concentrative sodium-dependent nucleoside transport activity with pyrimidine nucleoside-selective characteristics (18) except that guanosine was also a permeant. Two concentrative transporter proteins (hCNT1, hCNT2) were identified by molecular cloning using cDNAs prepared from human kidney (19, 43, 44, 52). It is uncertain whether the third human concentrative transporter (hCNT3) is also present in human kidney since hCNT3 mRNA levels detected in renal tissues in a commercial Northern blot were very low (41).

Asymmetric distribution of various transporters on cell surfaces is thought to determine the net absorption or secretion of nucleosides across epithelia. For example, it has been proposed that absorption of nucleosides in the gastrointestinal tract is accomplished by sodium-dependent concentrative transporters on apical surfaces and equilibrative transporters on basolateral surfaces, resulting in the net transport of dietary nucleosides from the intestinal lumen into blood (8). A similar hypothesis has been proposed for renal reabsorption of nucleosides by proximal tubules (35).

Information on the distribution and localization of nucleoside transporter proteins in human kidney will facilitate understanding of renal nucleoside reabsorption and excretion processes. In this paper, we describe results from studies of five of the seven known human nucleoside transporters (hENT1/2, hCNT1/2/3) in human kidney sections by RT-PCR and immunoblotting. Although mRNA encoding all five transporters was observed, hENT1 and hCNT3 were the major proteins detected by immunoblotting with monoclonal antibodies directed against each of the five human transporter proteins. The anatomic locations of hENT1 and hCNT3 were determined in human kidney sections, respectively, by immunohistochemistry and immunofluorescence using established marker proteins to identify proximal tubules, loops of Henle, and collecting ducts. Equilibrium binding studies were undertaken with the potent and highly specific inhibitor of hENT1, NBMPR, to confirm the presence of hENT1 on apical surfaces of proximal tubular membranes. Finally, uptake of [3H]uridine by polarized primary cultures of human renal proximal tubule cells (hRPTCs) was measured in the presence and absence of sodium and/or inhibitors of ENT-mediated transport (NBMPR, dilazep) to functionally identify nucleoside transport processes.

MATERIALS AND METHODS

Radioisotopes and antibodies. [3H]NBMPR (5.5 Ci/mmol at 98% purity) and [5-3H] uridine (40 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA) and were used without further purification. Ecollite was purchased from MP Biomedical (Irvine, CA). Monoclonal antibodies specific for the five nucleoside transporters considered in this study were developed and characterized previously (11, 21, 34) using synthetic peptides derived from hENT1, hENT2, hCNT1, hCNT2, or hCNT3 that corresponded to amino acids 254-271, 261-280, 31-55, 30-51, and 45-69, respectively. Other antibodies used in this study were: anti-human proximal nephrogenic renin antigen (PNRA; Zymed Laboratories), anti-human Tamm-Horsfall protein (THP; Cedarlane Laboratories, ON, Canada), anti-rat aquaporin-2 (AQP2; Alpha Diagnostics), and anti-human vascular type H+-ATPase B1/2 (V-ATPase; Santa Cruz Biotechnology). Iso-type control antibodies (mouse anti-IgG1k, mouse anti-IgG2b, mouse anti-IgM, and rabbit anti-IgG) were purchased from Sigma (St. Louis, MO) and horseradish peroxidase-conjugated dextran polymer (Dako EnVision+) was obtained from Dako (Carpentina, CA). Anti-mouse horseradish peroxidase secondary antibodies were from Jackson ImmunoResearch laboratories (West Grove, PA). Anti-mouse ZO1 antibodies were from Zymed Laboratories and were used to monitor the expression of the tight junction protein ZO1 in confluent monolayer cultures. AlexaFluor 488 and AlexaFluor 546 goat anti-mouse IgM secondary antibodies were obtained from Invitrogen (Burlington, ON, Canada).

Ethics and approval. The study was approved by the Institutional Review Board of the Alberta Cancer Board and by the University of Alberta/Canadian Health Research Ethics Board and informed consent was obtained from all patients.

Tissue source. Normal parts of human kidney were obtained from nephrectomized patients (10) with renal cell carcinoma. The outer cortex (RT-PCR and medulla were removed; tissue was cut into small pieces, washed twice with an ice-cold solution of PBS, containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM NaHPO4, and 1.47 mM KH2PO4 adjusted to a final pH of 7.4, to remove blood. A portion of each kidney preparation was formalin-fixed and paraffin-embedded for immunohistochemistry studies. Unfixed portions of kidney were used for immunofluorescence studies and for preparation of BBMV, crude membranes, and primary cultures of hRPTCs.

Total RNA isolation and RT-PCR. Total RNA was isolated from 0.1 g of normal human kidney cortex or medulla tissue using a GenElute Mammalian Total RNA Kit from Sigma. Total RNA was treated with DNase I (Invitrogen) before RT-PCR using Superscript One-Step RT-PCR with Platinum Taq (Invitrogen). Oligonucleotides used for hENT1, hENT2, hCNT1, hCNT2, and hCNT3 amplification were hENT1: 5'-gccttggaaccggcggeg-3' and 5'-tggagaagccaaagcgcaac-3'; hENT2: 5'-ttcagccgctagctaaag-3' and 5'-ggacggccccggacgcag-3'; hCNT1: 5'-ctgtggctgccctacccttcctg-3' and 5'-ggagggccccgccccgcaccc-3'; hCNT2: 5'-ccagcagcagctagctaaag-3' and 5'-ggacctggagttcgatgggagt-3'; hCNT3: 5'-ggacactgattcctacccggag-3' and 5'-ggggggctggagttcagttcgatgggagt-3'. PCR reactions in which DNA identities of amplified products were confirmed by DNA sequencing shown) Superscript II Reverse Transcriptase and Platinum Taq DNA Polymerase, 0.2 mM of each dNTP, 1.2 mM MgSO4, 0.2 μM of each forward and reverse oligonucleotides, and autoclaved distilled water to 50 μl. The reaction mixtures were heated to 45°C for 30 min, then 94°C for 2 min for cDNA synthesis. PCR amplification conditions were as follows for 40 cycles: 94°C for 1 min; 55°C for 1 min for hENT1 and hENT2 amplification, or 50°C for 1 min for hCNT1 and hCNT2 amplification, or 52°C for 1 min for hCNT3 amplification; and 72°C for 1 min. Afterwards, PCR reactions were heated to 72°C for 15 min and cooled to 4°C. Samples were then run in a 1.2% agarose gel (0.8 mM Tris-acetate, 0.04 mM Na2EDTA, pH 8.5; 0.5 μg/ml ethidium bromide). The expected sizes of the PCR products were 0.50 kb for hENT1, 0.43 kb for hENT2, 0.80 kb for hCNT1, 0.61 kb for hCNT2, and 0.48 kb for hCNT3. PCR reactions in which DNA template was substituted with water served as negative controls. Identities of amplified products were confirmed by DNA sequencing of excised bands. PCR amplification positive controls were performed on plasmid (pYPGE15) constructs that contained either hENT1, hENT2, hCNT1, hCNT2, or hCNT3 full-length inserts produced as previously described (51, 57).

Preparation of crude membranes. Crude membranes from kidney cortical and medulla samples were prepared as described previously using tissue from four kidneys (47). Tissues were minced into small pieces and washed thoroughly with PBS, pH 7.4, to remove blood. They were then suspended in ice-cold homogenization buffer (10 mM Tris·HCl, pH 7.5, 250 mM sucrose, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride) and homogenized. Homogenates were centrifuged (320 g, 10 min) at 4°C, and supernatants were
recentrifuged (15,000 g, 30 min) at 4°C. Pellets consisting of crude plasma membrane fractions were suspended in homogenization buffer and snap-frozen in liquid nitrogen before storage at −80°C until further use.

**Immunoblotting analyses.** Yeast membranes were prepared by a method described previously (50). Briefly, yeast cells producing recombinant hENT1, hENT2, hCNT1, hCNT2, or hCNT3 proteins were grown, cells were lysed, and membrane fractions were obtained by centrifugation of lysates at 120,000 g for 60 min. The resulting membrane pellets were resuspended in buffer that contained protease inhibitors. The samples were either used immediately or frozen at −80°C.

Crude membranes (20 μg protein each) from kidney tissues prepared as described above were subjected to SDS-polyacrylamide gel electrophoresis, after which proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA). The PVDF membranes were incubated overnight at 4°C in 0.2% Tween 20, Tris-buffered saline containing 5% (wt/vol) skim milk powder (blocking buffer) followed by incubation with monoclonal antibodies against either hENT1, hENT2, hCNT1, hCNT2, or hCNT3 in blocking buffer for 1 h at room temperature. PVDF membranes were washed three times with blocking buffer and incubated with anti-mouse horseradish peroxidase secondary antibodies in blocking buffer for 1 h and washed extensively with blocking buffer to remove unbound antibodies. Immunoreactive bands were visualized on X-ray film by enhanced chemiluminescence with horseradish peroxidase-conjugated anti-mouse IgG antibodies and cyclic diacylhydrazides (ECL, Amersham Pharmacia Biotech, Uppsala, Sweden). Protein content was determined with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

**Isolation of BBMV.** BBMV from kidney cortex were prepared as described previously (5). The final pellet of BBMV was resuspended in 300 mM mannitol, 5 mM Tris-HCl buffer, pH 7.4, and passed through a fine needle to produce uniform membrane vesicles. All procedures were carried out at 4°C. Alkaline phosphatase (EC 3.1.3.1) activity was monitored using the Sigma alkaline phosphatase activity kit (Sigma) to quantify enrichment of BBMV and protein concentrations were determined using the Bio-Rad protein assay kit. Portions of BBMV suspensions were snap-frozen in liquid nitrogen and stored at −80°C until further use.

**NBMPR binding.** B\textsubscript{max} and K\textsubscript{D} values for binding of NBMPR to hENT1 were obtained from mass law analysis of equilibrium binding data as described previously (50). BBMV were incubated with graded concentrations (0.12–8 nM) of [3H]NBMPR for 45 min to ensure that equilibrium between free and bound ligand was reached in the presence or absence of excess (10 μM) unlabeled NBMPR in 20 mM Tris, 3 mM K\textsubscript{2}HPO\textsubscript{4}, 144 mM NaCl, 1 mM MgCl\textsubscript{2}, 1.4 mM CaCl\textsubscript{2}, pH 7.4 (binding buffer). At the end of incubations, BBMV were collected on Whatman GF/B filters (Fisher Scientific Canada, Nepean, ON, Canada) under vacuum and filters were washed with ice-cold binding buffer. Filter-bound [3H]NBMPR was measured by scintillation counting. The amount of [3H]NBMPR that bound specifically was calculated as the difference between the amount of [3H]NBMPR that bound in the absence of 10 μM NBMPR and the amount that bound in its presence. Specific binding was plotted as a function of free NBMPR concentrations and subjected to mass law analysis to obtain B\textsubscript{max} and K\textsubscript{D} values. Data were analyzed by nonlinear regression using GraphPad Prism, version 3.0, software.

**Immunohistochemistry.** Immunohistochemistry was performed as described elsewhere (31, 33, 34, 45). Sections (4–6 μm) of formalin-fixed, paraffin-embedded kidney tissue were dried in an oven at 59°C for 2 h. Sections were rehydrated and, after antigen retrieval and blocking of endogenous peroxidase, sections were incubated at room temperature with primary anti-hENT1 monoclonal antibodies in PBS in a humidified chamber for 30 min. Sections were rinsed in PBS (pH 7.2), immersed in PBS for 5 min, and incubated with DAKO En Vision+ goat anti-mouse dextran conjugate PBS for 30 min. After being washed in PBS for 5 min, sections were incubated with diaminobenzidine solution, rinsed, and counterstained with hematoxylin. Staining for proximal nephrogenic renal antigen (PNRA), Tamm-Horsfall protein (THP), aquaporin-2 (AQP2), and human vacuolar type H\textsuperscript{+}-ATPase (V-ATPase) was performed according to each manufacturer’s instructions.

To identify segments positive for hENT1, consecutive sections were stained for defined tubule markers as previously described (22). Each tissue section that was stained for tubule markers was flanked by a consecutive tissue section that was stained for hENT1. Sections were incubated with appropriate dilutions of primary antibodies (protein μg/ml): 10 μg/ml for anti-hENT1 monoclonal antibodies; 0.3 μg/ml for anti-PNRA monoclonal antibodies; 4 μg/ml for anti-AQP2 polyclonal antibodies; 0.2 μg/ml for anti-H\textsuperscript{+}-ATPase polyclonal antibodies; and 0.5 μg/ml for anti-THP monoclonal antibodies. Controls for monoclonal antibodies were isotype mouse anti-IgG antibodies and controls for polyclonal antibodies were rabbit anti-IgG antibodies at appropriate dilutions. Slides with anti-hENT1 antibodies were incubated in a humidified chamber overnight at 4°C, whereas slides with all other antibodies were incubated for 30 min at room temperature. Sections were then rinsed with PBS, immersed in PBS for 5 min, incubated with goat anti-mouse dextran conjugate or goat anti-rabbit dextran conjugate (DAKO Envision+) for 30 min, followed by soaking in PBS. DAKO diaminobenzidine liquid chromagen was placed on samples for 5 min and rinsed with tap water, after which slides were soaked in 1% CuSO\textsubscript{4} for another 5 min, rinsed with tap water, counterstained with hematoxylin, dehydrated through graded alcohol and xylene. Immunohistochemistry studies were conducted with tissue samples obtained from four different kidneys. Slides were imaged using a Zeiss Axioskop2 plus Microscope (Carl Zeiss MicroImaging, Thornwood, NY) equipped with an F Fluar ×40/1.3 oil immersion lens and Zeiss Axiovision Software 3.1. Images for consecutive tissue sections stained for specific tubule markers and hENT1 were collected so that the images obtained contained the same kidney tubules from consecutive sections.

**Immunofluorescence staining.** Kidney tissue specimens were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA) and snap-frozen in a dry ice-methanol bath. Cryostat sections (4- to 6-μm thick) were picked up on glass microscope slides and dried at room temperature overnight, followed by a 10-min fixation in acetone, and then air-dried for 5 min. Immunofluorescence staining of frozen kidney tissue sections was performed in a humidified atmosphere using 3MM paper soaked in PBS in a rectangular Petri dish. The tissue was blocked for 30 min with 2% goat serum in PBS. hCNT3 monoclonal antibodies (50 μg/ml) in PBS were added to the tissue for 30 min at room temperature and washed three times with PBS (5 min/wash) using a Coplin jar. AlexaFluor 488 goat anti-mouse IgM secondary antibodies (8 μg/ml) PBS were added to slides for 30 min at room temperature, stored in a dark environment, and then washed three times with PBS (5 min/wash). All slides were mounted with a no. 1 coverslip using Mowiol mounting medium with p-phenylendiamine as anti-fade and with 1 μg/ml 4′-6-diamidino-2-phenylindole. To test anti-hCNT3 antibodies for specificity, 500 μg/ml of the antigenic peptide REHTNTKQDEEQVTVEQDSPRNREH or a unrelated peptide were added to anti-hCNT3 antibody solutions and incubated for 30 min at room temperature before they were applied to tissue sections.

Double immunofluorescence labeling of frozen kidney tissue sections for hCNT3 and either PNRA, THP, AQP2, or H\textsuperscript{+}-ATPase were performed by sequential incubations of antibodies in PBS: anti-PNRA antibodies (0.3 μg/ml), anti-THP (5 μg/ml), anti-AQP2 (8 μg/ml), and anti-H\textsuperscript{+}-ATPase (4 μg/ml) followed by AlexaFluor 488 goat anti-mouse secondary antibodies (8 μg/ml), followed by anti-hCNT3 antibodies (50 μg/ml), followed by AlexaFluor 488 goat anti-mouse IgM secondary antibodies. Controls for doublelabeling experiments included
replacement of either or both of anti-PNRA and anti-hCNT3 primary antibodies with the appropriate isotype control antibodies. Labeled cells were viewed on a Zeiss laser-scanning confocal microscope (LSM 510 version 3.2, Carl Zeiss MicroImaging) mounted on an Axiovert 100M inverted microscope with a plan Neofluar ×40/1.3 oil immersion lens. Argon and helium-neon (HeNe) lasers were sequentially used to scan at wavelengths of 488 and 543 nm, respectively. A UV laser (364 nm) was used to excite 4′,6-diamidino-2-phenylindole-stained cells. Images were collected according to Nyquist sampling with a 560-nm long-pass filter for Cyamine-3 signals, a 505- to 550-nm band-pass filter for Alexa 488 signals, and a band-pass filter of 385–470 nm for 4′,6-diamidino-2-phenylindole signals. Sections from four different kidneys were used for staining with hCNT3 antibodies in immunofluorescence studies.

Culture of hRPTCs. hRPTCs were isolated by the enzyme dissociation method using a collagenase-DNAase mixture as described elsewhere (4). Isolated hRPTCs were cultured on collagen (Inamed Biomaterials, Fremont, CA)-coated plastic surfaces in a serum-free mixture of DMEM-Ham's F-12 medium (50:50 by volume, Invitrogen) with the following additions per l: selenium (5 μg), insulin (5 mg), transferrin (5 mg), hydrocortisone (36 μg), epidermal growth factor (10 μg, BD Biosciences), triiodothyronine (4 ng, Sigma), and 2 mM glutamine (GIBCO). Confluent monolayers of hRPTCs incubated at 37°C in a humidified atmosphere containing 5% CO2 were subcultured by detaching with trypsin-EDTA (0.5 and 0.2 g/l, respectively). For transport studies, hRPTCs were seeded at confluence into 12-well tissue culture plates coated with collagen and incubated for 5–7 days at 37°C in a humidified atmosphere containing 5% CO2.

Nucleoside uptake assays. Uridine uptake by confluent hRPTCs was measured as described previously (14) by incubating replicate cultures grown in 12-well tissue culture plates with 1 μM [3H]uridine (1 μCi/ml) in sodium buffer (144 mM NaCl, 3 mM K2HPO4, 1.2 mM CaCl2, 1 mM MgCl2, 20 mM Tris-HCl, and 5 mM d-glucose) or sodium-free buffer (144 mM N-methyl-β-glucamine (NMDG)), 3 mM K2HPO4, 1.2 mM CaCl2, 1 mM MgCl2, 20 mM Tris-HCl, and 5 mM d-glucose in the presence or absence of 10 mM unlabeled uridine, 1 mM unlabeled thymidine, 1 mM unlabeled inosine, 200 μM dilazep, and/or 0.1 μM NBMPR. After 10 min of incubation, plates were washed with ice-cold sodium or sodium-free buffer three times. Cells were lysed with 5% Triton X-100 for 1 h and transferred to liquid scintillation vials containing 10 ml EcoLite liquid scintillation fluid (MP Biomedicals, Solon, OH). Radioactivity was counted using a Beckman LS 6500 multipurpose scintillation counter (Mississauga, ON, Canada).

RESULTS

Expression of mRNAs encoding hENTs and hCNTs in human kidney. To assess expression of nucleoside transporter genes in human kidney cortex and medulla, RT-PCR was performed using specific primers for hENT1/2 and hCNT1/2/3. As resolved by agarose gel electrophoresis and visualized by ethidium bromide staining, reaction products of predicted sizes were amplified from first-strand cDNA for the five transporter types. RT-PCR analysis of kidney samples from two different individuals identified transcripts for hENT1, hENT2, hCNT1, hCNT2, and hCNT3 in both cortex and medulla (Fig. 1). Genomic DNA contamination was not detected in RT-negative PCR reactions. DNA sequencing of excised bands confirmed the identities of amplified products. The same results were obtained in experiments from human kidney cortex and medulla samples from four additional individuals (data not shown).

Identification of hENTs and hCNTs by immunoblotting. Immunoblotting analyses with monoclonal antibodies specific for hENT1, hENT2, hCNT1, hCNT2, or hCNT3 were carried out with crude membrane fractions (20 μg protein each) prepared from human kidney cortex and medulla of three different individuals. Membrane fractions prepared from yeast producing either recombinant hENT1, hENT2, hCNT1, hCNT2, or hCNT3 proteins were also loaded as controls. The results for hENT1 and hCNT3 are shown in Fig. 2. Major immunoreactive proteins at 45–48 and <90 kDa, corresponding to hENT1 and hCNT3, respectively, were detected in both cortex and medulla in all three samples (Fig. 2). hCNT3 migrated lower than 90 kDa, whereas the recombinant nucleoside transporter protein controls migrated at slightly higher than 90 kDa. In contrast, little, if any, hENT2, hCNT1, or hCNT2 was detected at the same protein levels (although the yeast controls were positive), indicating that hENT2, hCNT1, or hCNT2 were either absent or below limits of detection of immunoassays (data not shown). The lower molecular weight immunoreactive bands in the hENT1 (top) panel could have been due to incomplete glycosylation of the hENT1 protein or to proteolysis during storage of membrane fractions.

Localization of hENT1 and hCNT3 in human kidney. Since the immunoblotting results suggested that hENT1 and hCNT3 proteins were more abundant than hENT2, hCNT1, or hCNT2 in kidney tissue membranes, localization studies were undertaken to assess the anatomic distribution of hENT1 and hCNT3. hENT1 was examined using immunohistochemistry methods and hCNT3 using immunofluorescence methods since results from immunohistochemistry studies of hCNT3 were not clear-cut, possibly due to the effects of paraformaldehyde fixation of kidney (20). Frozen tissue sections fixed with acetone postsectioning retained good immunoreactivity for hCNT3. Kidney samples from four different individuals yielded similar results for both transporters.
Validation of the specificity of the antibodies. Specificity of the anti-hENT1 and anti-hCNT3 antibodies was confirmed in peptide competition assays. Antibodies against hENT1 stained apical surfaces of tubules (Fig. 3A), whereas preadsorption of anti-hENT1 antibodies with excess immunogenic peptides corresponding to amino acids 254-271 of hENT1 abolished positive staining (Fig. 3C) and preadsorption with excess hCNT3-specific immunogenic peptides corresponding to amino acids 45-69 of hCNT3 had no effect (Fig. 3B). Similarly, the specificity of hCNT3 immunofluorescent staining (Fig. 3D) was verified by retention of staining when anti-hCNT3 antibodies were preadsorbed with excess hENT1 peptides (Fig. 3E) and loss of staining when anti-hCNT3 antibodies were preadsorbed with hCNT3 antigenic peptides (Fig. 3F).

Proximal tubules. Immunohistochemistry and immunofluorescence staining were undertaken to compare distributions of nucleoside transporter proteins hENT1 and hCNT3, which had been detected by immunoblotting of crude membranes. Positive staining for hENT1 was detected in proximal tubules of the kidney cortex (Fig. 4, A and B). PNRA strongly labeled brush-border regions of proximal tubules (Fig. 4C), and a comparison of the PNRA and hENT1 staining in consecutive kidney sections of the same specimens revealed that hENT1 was concentrated in PNRA-defined proximal tubules. The background staining observed in the proximal tubular lumen was likely due to the presence of cellular debris from brush borders of proximal tubules. Positive staining for hCNT3 in proximal tubules was demonstrated by confocal microscopy with double immunofluorescence staining with antibodies against hCNT3 (Fig. 4E) and PNRA (Fig. 4F) and counterstaining with 4’-6-diamidino-2-phenylindole, which stains nuclei. hCNT3 staining was intense toward apical aspects of membranes with some intracellular staining as well (Fig. 4H). Panel I shows the negative control in these studies. The merged images (Fig. 4, G and H) showed colocalization of the red and green signals of PNRA and hCNT3, respectively, on apical surfaces of proximal tubules, although some intracellular staining of hCNT3 was also present.

Thick ascending loop of Henle. THP is present in the thick ascending loops of Henle and immunohistochemistry with antibodies against hENT1 and THP in consecutive kidney sections of the same specimens showed moderate apical and basolateral hENT1 surface staining (Fig. 4, I and J) in THP-defined (Fig. 4K) thick ascending loops of Henle. Double immunofluorescence staining with antibodies against hCNT3 (Fig. 4M) and THP (Fig. 4N) and counterstaining with DAPI showed localization of CNT3 in THP-stained tubules (Fig. 4, O and P) and apical surface staining of hCNT3 (Fig. 4, M, O, P) in thick ascending loops of Henle.

Collecting ducts. Immunohistochemistry with antibodies against hENT1 and two markers of collecting ducts (AQP2, H^+-ATPase) in consecutive tissue sections of the same specimens showed moderate apical and basolateral surface localization of hENT1 (Fig. 5, A, B, I, J) in collecting duct cells identified by both AQP2 (Fig. 5C) and H^+-ATPase staining (Fig. 5K). In contrast, double immunofluorescence staining with antibodies against hCNT3 (Fig. 5, E, F and M) and either AQP2 (Fig. 5F) or H^+-ATPase (Fig. 5N) showed no detectable staining of hCNT3 in collecting ducts defined by either AQP2 (Fig. 5F) or H^+-ATPase (Fig. 5N), indicating absence of hCNT3 in collecting ducts.

NBMPR binding analyses. [^3H]NBMPR binding to BBMV prepared from human kidney cortex was used to functionally confirm the presence of hENT1. hENT1 binds NBMPR with high affinity (<=1 nM) and equilibrium-binding studies of [^3H]NBMPR with BBMV were conducted to obtain a measure of hENT1 abundance. Specific binding of [^3H]NBMPR to BBMV was saturable (Fig. 6) and, since the Scatchard plot was linear (Fig. 6, inset), a one-site binding model was used to estimate B_max (3.01 ± 0.05 pmol/mg protein) and K_d (0.22 ± 0.02 nM) values for binding. The B_max value yielded an estimated 1.8 x 10^12 hENT1 sites/mg BBMV protein.

Identification of NT activities in primary cultures of proximal tubule cells. Since immunostaining experiments showed that hENT1 and hCNT3 were present in human kidney proximal tubules, experiments were undertaken to functionally identify hENT1 and hCNT3 in hRPTCs. Normal regions of human kidney tissues obtained from patients undergoing nephrectomy for localized renal cell carcinoma were used to generate hRPTCs by established methods (4). hRPTCs were subcultured up to eight generations in serum-free media on collagen-coated surfaces under conditions for which they retained phenotypic characteristics of human kidney proximal tubules including 1) expression of brush-border enzymes acid phosphatase, alkaline phosphatase, and γ-glutamyl transferase, 2) the presence of sodium-dependent glucose transport activity, and 3) the responsiveness to parathyroid hormone (1). Confluent monolayers of hRPTCs that were used for uridine uptake studies developed tight junctions as assessed by anti-ZO-1.
staining (data not shown), confirming that the cultures were polarized.

To identify concentrative and equilibrative nucleoside transport processes present in hRPTCs, uptake of radiolabeled uridine was measured under conditions that eliminated either equilibrative or concentrative nucleoside transport processes. Uptake mediated by hCNTs was assessed in sodium-containing buffer with 200 μM dilazep to inhibit equilibrative transport activity. Under these conditions, uptake of 1 μM [3H]uridine was completely inhibited by 10 mM uridine, 1 mM thymidine, or 1 mM inosine (Fig. 7A), indicating that the dominant concentrative nucleoside transporter in hRPTCs was the broadly selective hCNT3. The higher uptake (P <0.001) observed in the presence of dilazep than in its absence presumably reflected blockade by dilazep of uridine efflux via hENT-mediated processes. hENT-mediated uptake of 1 μM [3H]uridine was determined in sodium-free buffer in the absence or presence of 0.1 μM NBMPR or 200 μM dilazep and is defined as that component of uptake that is inhibited by 10 mM uridine. The almost complete inhibition of mediated uridine uptake by NBMPR indicated that the dominant equilibrative nucleoside transporter in hRPTCs was hENT1 (Fig. 7B). The small component of uptake that was not inhibited by 0.1 μM NBMPR but was inhibited by 200 μM dilazep was probably mediated by hENT2. The net mediated nucleoside uptake activities of the three transporters are shown in Fig. 7C.

DISCUSSION

Functional studies in animal kidney cells and in membrane preparations from animal and human kidneys have shown multiple sodium-dependent and sodium-independent transport systems in renal epithelia (18, 29, 31, 54–56). Although CNTs seem to be restricted to apical membranes of polarized cells (18, 53), the location of ENTs is controversial and hENT1 and hENT2 (es and ei activities since hENT1 and hENT2 were not cloned at that time) have been reported in apical (48), basolateral (55), or in both apical and basolateral membranes (12) of kidney cells. More recently, when recombinant fluorescently tagged proteins were produced in cultured renal epithelial cells, hENT1 was found on both apical and basolateral membranes, whereas hENT2 was found only on basolateral membranes (37). In contrast, recombinant fluorescently tagged hCNTs have been found only in apical regions of cultured renal cells (26, 35). Earlier studies identified mRNA transcripts for the five plasma membrane nucleoside transporters in human kidney by RT-PCR and Northern blotting (40, 42).

Both ENTs and CNTs have been suggested to play important roles in renal tubular reabsorption and secretion of nucleosides and nucleoside drugs in humans (35). However, to date, there is no information regarding the precise anatomic locations of nucleoside transporter proteins in human kidney. The present study was undertaken to determine the abundance and distribution of nucleoside transporters in renal cortex and medulla of normal portions of human kidneys removed from patients with renal carcinoma.

As an initial step, we evaluated expression of mRNA encoding various nucleoside transporters using RNA isolated from cortex and medulla of human kidney specimens from five different patients. RT-PCR analysis of RNA samples identified transcripts for hENT1, hENT2, hCNT1, hCNT2, and hCNT3 in
Fig. 4. Localization of hENT1 and hCNT3 in proximal tubules and loops of Henle of human kidney by immunohistochemistry and immunofluorescence. A–D: hENT1 and PNRA immunohistochemistry in human kidney cortex. hENT1 localizes to brush-border membranes of proximal tubules (A and B), indicated by the arrow heads. *PNRA-negative tubule that has a distinctly different hENT1 staining pattern with apical and basolateral membrane staining. Proximal tubule marker PNRA localizes to brush-border membranes of proximal tubules (C), indicated by the arrow head. Isotype control staining for hENT1 and PNRA are negative (D). E–H: hCNT3 and PNRA double immunofluorescence in human kidney cortex. hCNT3 in green (E) and proximal tubule marker PNRA in red (F) colocalize to brush-border membranes of proximal tubules in yellow (merge; G, H), indicated by the arrow heads. The arrow (→) indicates intracellular staining of hCNT3 in proximal tubule cells. I–K: hENT1 and THP immunohistochemistry in serial sections of human kidney. hENT1 localizes to apical (arrow head) and basolateral (←) membranes of loops of Henle (I, J). Loop of Henle marker THP localizes to apical membranes and lumen of loops of Henle (K). *THP-negative tubule that has hENT1 staining. L–P: hCNT3 and THP double immunofluorescence in human kidney. hCNT3 in green (M) and loop of Henle marker THP in red (N) localize to apical membranes of loops of Henle (merge; O, P), indicated by arrow heads. *THP-positive tubule with no hCNT3 staining. The arrow (→) indicates intracellular staining of hCNT3 (P). Isotype control staining for hCNT3 and THP is negative (L). Hematoxylin (immunohistochemistry) and 4’-6-diamidino-2-phenylindole (immunofluorescence) counterstains for nuclei are shown in blue. Scale bars shown are 50 μm.
Collecting Ducts

Fig. 5. Localization of hENT1 and hCNT3 in collecting ducts of human kidney by immunohistochemistry and immunofluorescence. A–D: hENT1 and AQP2 immunohistochemistry in serial sections of human kidney medulla. hENT1 localizes to apical (arrow head) and basolateral (→) membranes (A, B) of collecting ducts denoted by the *. AQP2, a collecting duct marker, localizes to apical membranes of collecting ducts (C), indicated by arrow head. Isotype control staining for hENT1 and AQP2 are negative (D). E–H: hCNT3 and AQP2 immunofluorescence staining in human kidney medulla. Double immunofluorescence with hCNT3 in green (E) and AQP2 in red (F) shows absence of hCNT3 staining in AQP2-stained collecting ducts (G). Principal cell marker AQP2 in red localizes to apical membranes of collecting duct principal cells (F, H). *AQP2-positive tubule with no hCNT3 staining. I–K: hENT1 and H\(^+\)-ATPase immunohistochemistry. hENT1 localizes to apical (arrow head) and basolateral (→) membranes of the collecting ducts (*; I, J). Collecting duct marker H\(^+\)-ATPase localizes intracellularly in collecting duct intercalated cells (K). The arrow head indicates an intercalated cell. L–P: hCNT3 and H\(^+\)-ATPase immunofluorescence in human kidney medulla. Double immunofluorescence with hCNT3 in green (M) and H\(^+\)-ATPase in red (N) shows absence of hCNT3 staining in collecting duct intercalated cells, merge (O, P). Collecting duct marker H\(^+\)-ATPase in red localizes to intracellular membranes of collecting duct intercalated cells (N, P). *Collecting duct containing intercalated cells with H\(^+\)-ATPase staining indicated by the arrow head. Isotype control staining for hCNT3 and H\(^+\)-ATPase is negative (L). Hematoxylin (immunohistochemistry) and 4\,-6-diamidino-2-phenylindole (immunofluorescence) counterstains for nuclei are shown in blue. Scale bars shown are 50 \(\mu\)m.
both cortex and medulla of all specimens, indicating expression of all five transporter genes. Crude membranes from both cortex and medulla from four different kidney specimens were prepared and evaluated by immunoblotting using transporter-specific antibodies. hENT1 and hCNT3 were detected in all four samples, whereas little, if any, hENT2, hCNT1, or hCNT2 was detected, indicating that the latter were either absent or below limits of detection of the immunoassays. The abundance of hENT1 and hCNT3 varied between samples and between the cortex and medulla of each sample.

Analysis of hENT1 abundance by mass-law analysis of [3H]NBMPR binding to BBMV prepared from the cortical region of a single human kidney specimen verified the presence of apical hENT1 in kidney cortical membranes. The specific activity of [3H]NBMPR binding was of a magnitude that could not be accounted for by residual red blood cell contamination of the kidney samples used to prepare the BBMV (25).

Subsequent immunolocalization in renal cortex and medulla of hENT1 and hCNT3 proteins, respectively, in paraffin sections by immunohistochemistry or frozen sections by immunofluorescence was carried out using monoclonal antibodies specific for either hENT1 or hCNT3. In normal human kidney, PNRA staining is localized to apical surfaces of proximal tubules (22), and in this work hENT1 staining in PNRA-defined proximal tubules of consecutive human kidney sections was observed only on apical surfaces. Prominent hCNT3 staining on apical surfaces of proximal tubules was evident in immunofluorescence studies and was colocalized with PNRA staining. In addition, some intracellular hCNT3 staining was observed in proximal tubules. In peptide preadsorption experiments, the immunogenic hENT1 and hCNT3 peptides completely abolished specific staining obtained with hENT1 and hCNT3 antibodies, respectively, whereas nonspecific peptides were ineffective, demonstrating that the staining observed was epitope specific.
Thick ascending loops of Henle of normal human kidney, which were identified by staining of THP, a specific loop of Henle marker (22), exhibited moderate apical and basolateral surface staining of hENT1 and apical surface staining of hCNT3. Collecting duct cells, which were identified by both AQ2p and H\(^+\)/ATPase staining (22), exhibited moderate apical and basolateral hENT1 staining and no detectable hCNT3 staining.

The observed differences in ENT1 localization in the current study and previously published data in polarized epithelial kidney cell culture models (26, 35–37) may be due to differences in experimental approaches, specifically localization of endogenous transporters in human kidney tissue specimens vs. recombinant transporters in transfected animal kidney-derived cell lines. For example, overexpression of recombinant fluorescent protein-tagged hENT1 may have saturated protein trafficking pathways, thereby giving rise to basolateral localization of hENT1 in transfected cells (26, 35–37). It is also possible, since those studies employed polarized cultures of a pig kidney LLC-PK1 cell line producing recombinant hENT1 (26, 35–37), that differences in localization between human kidney specimens and LLC-PK1 cells were species dependent. Finally, although the LLC-PK1 cells exhibit many characteristics of proximal tubules, they are responsive to antidiuretic hormone, a characteristic specific to collecting tubules (10).

CNT1 and CNT2 functional activities have been shown to be present in bovine kidney BBMV (56). Studies of rat (r) CNT1 protein in kidney localized rCNT1 predominantly on apical membranes of renal cortical tubules (20), consistent with its role in absorption of nucleosides. Although transcripts for all three hCNTs have been found in human kidney (this work and Refs. 15, 43, 44, 46, 52), the results presented here identified hCNT3 as the major CNT in human kidney; hCNT1 and hCNT2, if present, were not detected in immunoblotting experiments with crude membrane preparations. hCNT1/2 have Na\(^+\)-nucleoside coupling ratios of 1:1 and hCNT3 has a Na\(^+\)-nucleoside coupling ratio of 2:1 (42, 46). A physiological implication of this difference in coupling ratio is that hCNT3 is capable of utilizing the electrochemical Na\(^+\) gradient to generate a trans-membrane nucleoside concentration 10-fold higher than either hCNT1 or hCNT2 (15, 35), suggesting that hCNT3 may be the key transporter involved in renal reabsorption of nucleosides. In addition, hCNT3 but not hCNT1/2 exhibits pH-dependent transport activity and thus can function as an H\(^+\)-nucleoside cotransporter (46). The pH dependence of hCNT3 activity has important physiological relevance in the kidney since the lumen of the proximal renal tubule has an acidic pH (6). In terms of permeant selectivity, the CNT3 isoform is functionally equivalent to CNT1 + CNT2.

To assess the nucleoside transport capacity of proximal tubules, uptake of \([\text{H}]\)uridine by primary cultures of hRPTCs was determined under conditions that enabled identification of particular hENTs and hCNTs. When such cultures are grown to confluence (5–7 days) before flux experiments, cells maintain polarity and grow with the brush-border membrane facing upwards (27). The confluent hRPTCs used for uptake studies were polarized with apical tight junctions, so that measured fluxes were the result of uptake across brush-border membranes. hENT1 and hCNT3 were the major nucleoside transport activities in hRPTCs, consistent with the apical localization of these transporters in proximal tubules shown in this work. A minor transport activity, probably mediated by hENT2, was also observed in hRPTC cultures.

In summary, our results using gene expression, protein abundance, inhibitor binding, immunohistochemistry, immunofluorescence, and nucleoside uptake studies demonstrated hENT1 and hCNT3 to be the major nucleoside transporter proteins in human kidney. hENT1 was found on apical surfaces of proximal tubules and apical and basolateral surfaces of loop of Henle, distal tubules, and collecting ducts, whereas hCNT3 was found on apical surfaces of proximal tubules, loop of Henle, and distal tubules. Although these results are consistent with a primary role for hCNT3 in reabsorption of nucleosides from apical surfaces of proximal tubules, they suggest that hENT1, which was observed on apical, but not basolateral, surfaces, may modulate reabsorption, raising the possibility that hENT2 (or some other transporter type) moves nucleosides across basolateral surfaces of proximal tubules during reabsorption. Nucleoside transporters are likely involved in regulating renal levels of extracellular adenosine, which has a multiplicity of physiological and pathophysiological functions, including lowering of glomerular filtration rates, stimulating Na\(^+\) reabsorption in proximal segments, and inhibiting Na\(^+\) reabsorption in medullary segments (49).

ACKNOWLEDGMENTS

We acknowledge the help of Dr. G. Todd and Dr. M. Chetner, University of Alberta Hospital, in providing the human nephrectomy specimens.

GRANTS

This work was supported by the Research Initiatives Program of the Alberta Cancer Board. C. E. Cass is Canada Research Chair in Oncology. J. D. Young is Heritage Scientist of the Alberta Heritage Foundation for Medical Research. A. N. Elwi is supported by a studentship from the Translational Research Training in Cancer Program jointly supported by the Canadian Institutes of Health Research, the National Cancer Institute of Canada, and the Alberta Cancer Foundation. M. B. Sawyer is a recipient of an American Society of Clinical Oncology Career Development Award.

REFERENCES


