Concentrative Nucleoside Transporter 3 Is Located on Microvilli of Vaginal Epithelial Cells

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ABSTRACT: Transporters are specialized integral membrane proteins, which mediate the passage of virtually all molecules through cell membranes. They are expressed in a broad range of human and animal tissues and play important roles in both normal and disease states. For these reasons, they are evaluated when developing and testing drugs. Two major families of drug transporters, the adenosine 5′-triphosphate-binding cassette and solute carrier transporters (SLC), have critical roles in the absorption, distribution, metabolism, and elimination of drugs. The SLC family contains known nucleoside transporters and therefore are important when nucleoside analogs are used as drugs to prevent or treat viral infections. In this study, we wanted to determine if it was possible to locate one member of the SLC family, the human concentrative nucleoside transporter 3 (CNT3) in human vaginal epithelial cells. The CNT3 protein has important roles in drug delivery, subsequent drug tissue distribution, and, hence, efficacy. Vaginal epithelial cells, taken from two human volunteers (one Caucasian and one African American), were labeled for light and electron microscopy, with a commercial antibody to a cytoplasmic domain of CNT3, the protein product of the SLC28A3 gene. Fluorescent secondary antibodies or protein A-gold were used to detect antibody binding. By electron microscopy, gold particle binding was quantified to determine labeling specificity. By light microscopy, positive labeling with anti-CNT3 antibodies was detected on human vaginal epithelial cells, but specificity to any intracellular structure was not easily determined, most likely a result of specimen preparation. Electron microscopy revealed that the CNT3 transporter protein was present predominantly on microvilli located on one side of some human vaginal epithelial cells. Quantification confirmed specific anti-CNT3 labeling over human vaginal epithelial cell microvilli. The CNT3 protein, present in the microvilli of human vaginal epithelial cells, may have a role in redistributing nucleoside homologues delivered to the vaginal tract. Transporter proteins such as CNT3 could shuttle nucleosides and their analogs through the vaginal epithelium to immune cells located in lower cell layers. Outer layers of cells, which are eventually shed from the epithelium, may remove accumulated nucleoside drug analogs from the vaginal tract.

INTRODUCTION

Nucleoside analogs such as acyclovir, a broad acting antiviral, are useful for the prevention and treatment of sexually transmitted diseases caused by viruses, especially those residing in cells. Treatment of intracellular viruses depends on antiviral drugs being carried across cell membranes and accumulating within the cells. Transporters such as the human concentrative nucleoside transporter, CNT3 (a product of the SLC28A3 gene) play an important role in carrying nucleosides and their analogs into cells.

Epithelial cells host a variety of transporters that typically exhibit concentrative carriers at the apical side, and equilibrative carriers located at the basolateral membrane, allowing coupling to occur, permitting the absorption or elimination of solutes. CNT3 transporters mediate the transport of both purine and pyrimidine nucleosides, and they are involved in adenosine signaling regulation at the cellular level. The CNT3 protein has important roles in drug delivery, subsequent drug tissue distribution, and, hence, efficacy because of its ability to absorb, distribute, and eliminate drugs. While the expression of transporter proteins within various epithelial tissues is well-known, information on drug transporters in vaginal epithelial cells (VEC) remains scarce. Better comprehension of the abundance and localization of different drug transporters is critical for the development of efficient antimicrobial and antiviral drug delivery methods for women, which are dependent on adequate drug absorption and
distribution within the vaginal epithelium. Additionally, drug delivery systems, such as vaginal rings, are utilized worldwide for birth control, and more recently, pre-exposure prophylaxis (PrEP). Research that reveals the locations and exact mechanisms of drug transporter function is critical for efficient drug absorptions among vaginal ring users. The advancement of drug development depends on the optimization of various nucleoside analogs.

The aim of this study is to develop an approach for determining the location of drug transporters in VEC using microscopy and commercial antibodies. Comparisons between results obtained by light and electron microscopy using antibodies to CNT3, an uptake sodium-coupled nucleoside transporter abundant in epithelium, is presented. The approach, using a combination of immunocytochemical and quantitative methods, will assist in validating antibodies with general availability, and form the basis of detailed studies of transporter proteins in VEC and other cell types.

■ RESULTS

The anti-CNT3 antibodies label agarose-embedded VEC with a minimal nonspecific label (Figure 1). On some VEC, the fluorescent signal was located on one region of the cell membrane (Figure 1), with intracellular punctate labeling (Figure 1). Although the cells labeled with the antibodies, the CLSM imaging did not easily reveal any identifiable structural specificity within or on the cells, perhaps because of the scraped cells being disturbed during preparation.

The cytoplasmic location of the binding site of the anti-CNT3 antibody was confirmed by comparing CNT3 labeling on cells, which had not been treated with Triton X-100, with cells that had been treated with the detergent. Triton X-100 opens the membranes of aldehyde-fixed cells giving antibodies access to intracellular sites. As expected, cells not treated with Triton X-100 had only low levels of CNT3 labeling (Figure S1A), while treated cells labeled with the anti-CNT3 antibodies (Figure S1B).

Thin sections through VEC prepared for electron microscopy were labeled with specific anti-CNT3 antibodies. Antibody binding on the sections was visualized using particulate protein A-gold particles (PAG10) on thin sections. The PAG10 label, indicating CNT3 binding, was associated with microvilli (Figure 2) located on one face of the VEC (Figure 2 and Figure S2). The microvilli had a diameter of approximately 100 nm (Figure S2B). In addition to being present over the inner part of microvilli, the antibody labeling occurred in low frequency over the cell plasma membrane, cytoplasm, unidentified cytoplasmic structures, and intracellular cytokeratin bundles. The cytoplasmic structures, which were labeled, could not be identified as specific structures, but they all appeared to be membrane-bound vesicles.

Sections labeled with the anti-CNT3 antibodies and PAG10 were used to count the numbers of PAG10 particles observed over each identified compartment. Using a surveying protocol, we were able to show that between 40 and 60% of the gold particles on the sections were found over VEC microvilli profiles. Smaller numbers of PAG10 particles were found over the cell plasma membrane, cytoplasm, unidentified cytoplasmic structures, and intracellular cytokeratin bundles. The number of gold particles counted over support film ("background"), in regions where there were no sections, was in the same range as the numbers of PAG10 particles over the cytokeratin bundles. Plasma membrane, cytoplasm, and unidentified cytoplasmic structures all had numbers of associated PAG10 particles that were slightly above nonspecific labeling (Figure 3 and Table 1). No label was detected over nuclear profiles or mitochondria (data not shown).

Comparing the PAG10 counts on sections from two separate grids labeled simultaneously with anti-CNT3 and
Table 1. PAG10 Particle Counts and Data Used for Calculating the Pearson Correlation coefficient

| Grid | X | Y | XY |
|------|---|---|----|
| Grid 1 | 43 | 40 | 1849 | 1600 | 1720 |
| Grid 2 | 1 | 18 | 1 | 324 | 18 |
| Grid 3 | 2 | 14 | 4 | 196 | 28 |
| Grid 4 | 134 | 120 | 17,956 | 14,400 | 16,080 |

Table 2. Pearson Correlation Coefficients (r), Showing the Degree of Agreement between Gold Label on Different Grids

| Grid # | Pearson Correlation Coefficient (r) | 95% CI | p | Spearman’s ρ |
|--------|-----------------------------------|-------|---|---------------|
| 1–2    | 0.99                              | 0.918–0.991 | 0.0001 | 0.81 | p = 0.0498 |
| 3–4    | 0.94                              | 0.56–0.994 | 0.0046 | 0.61 | p = 0.2417 |
| 1–3    | 0.94                              | 0.549–0.997 | 0.0051 | 0.714 | p = 0.1361 |
| 1–4    | 0.83                              | 0.062–0.981 | 0.04 | 0.2 | p = 0.714 |
| 2–3    | 0.94                              | 0.53–0.993 | 0.0055 | 0.26 | p = 0.618 |
| 2–4    | 0.87                              | 0.187–0.985 | 0.0253 | 0.06 | p = 0.91 |

“Raw data of PAG10 particle counts from sections on four different specimen grids. Grids 1 and 2 were labeled together. Grid 3 and grid 4 were labeled together but on a different day. The table shows the number of PAG10 particles found over each identifiable structure on the sections. Columns labeled “x”, “y” and “xy” show the data from grids 1 (x) and 2 (y) processed for calculating the Pearson correlation coefficient (Table 2).”

PAG10 provided raw data (Table 1), which was used to obtain the Pearson correlation coefficient (r) between the grids. The r value for comparing the labeling on grids 1 and 2 was 0.99 (95% CI: 0.918–0.991, p = 0.0001), and the r value for grids 3 and 4 was 0.94 (95% CI: 0.549–0.994, p = 0.0051) (listed on Table 2). The probability of the distribution of labeling being uniform is <0.0001 for all comparisons, by the χ² test suggesting that there is a systematic labeling. The proportion of gold particles on microvilli is much greater than expected if the distribution were random.

Closer examination of the labeling pattern on the CNT3-labeled cells showed the cells had a polarity. Labeling was concentrated over microvilli on one part of the cell. The unlabeled parts of the cells also lacked microvilli (Figure S2). When the PAG10 particles were quantified using cross-lattice overlays, the association with microvilli was further supported (Figure 4). The number of gold particles on the plasma membrane of apical regions (identified by the presence of microvilli) were higher than the numbers of gold particles on the plasma membrane of basolateral surfaces (with reduced numbers of microvilli) (Figure 4). The number of PAG10 particles over microvilli on the apical surface was also higher than the PAG10 on microvilli on the basolateral surfaces (Figure 4). PAG10 particles closer than 3 nm to the membrane
were counted, as were the gold particles 3–5 nm distant from the membrane (Figure 4). On apical microvilli, the PAG10 particles were equally distributed between being close to the membrane (<3 nm) and being 3–5 nm away from the membrane (Figure 4). Apical plasma membrane labeling was mostly 3 nm or less from the membrane (Figure 4).

The anti-CNT3 labeling was not distributed uniformly over the microvilli. While the mean microvilli labeling was 8 gold particles per μm length (Figure 4), this estimate may be low because of the presence of microvilli on the apical surface with few PAG10 particles. The heterogeneous labeling pattern of microvilli was demonstrated by comparing similar membrane zones on sequential sections of individual cells. The same CNT3 labeled regions on microvilli showed a high frequency of label over microvilli in one section (Figure 4 and Figure S3) but reduced labeling on the sequential section (Figure 5 and Figure S3), even though both sections were mounted on the same specimen grid and labeled together.

Finally, in order to confirm the PAG10 labeling on sections was due to specific CNT3 binding and not due to protein A binding to cells, a PAG10 control experiment was performed. Two adjacent serial sections, each 60 nm thick, were mounted on grids and labeled. One was labeled with anti-CNT3 and PAG10. The other section was only labeled with PAG10. Each section was imaged and similar regions on identical cells were compared (Figure 6). The CNT3 antibodies labeled membranes, microvilli, and intracellular membranes (Figure 6A,C). Gold particles were not detected over the same structures on sections labeled only with the PAG10 (Figure 6B,D).

**DISCUSSION**

The CNT3 drug transporter protein was located on the microvilli of VECs using high-resolution immunolabeling. The CNT3 protein is a member of the solute carrier (SLC) transporters, and is primarily involved with carrying purine and pyrimidine nucleosides across biological membranes. The CNT3 protein can carry nucleoside analogs used in antiretroviral treatments across membranes, so has the potential to affect drug efficacy.

The aim of this work was to test a method for locating the human concentrative nucleoside transporter CNT3 in or on VEC from female volunteers. The VEC, from a healthy Caucasian volunteer in her mid 30s, were immunolabeled with a commercial antibody, which has previously been used to identify CNT3 in human pancreatic cancer cells by western blotting and immunofluorescence microscopy. Here, we show CNT3 is present on VEC, with a concentration of label over microvilli on one face of the cells. A second quantitative analysis was performed on VEC from an African America female. Cross lattice overlays on a random series of images were used to determine the PAG10 particle density on the
two different regions of labeled cells. The membranes with PAG10-positive microvilli were designated “apical”, and membranes without microvilli were designated “basolateral”, consistent with other reports that CNT proteins are restricted to apical regions of polarized cells. The CNT3 labeling on cells from the two females were similar, with most of the PAG10 being detected on microvilli.

By light microscopy, labeling experiments using the polyclonal anti-CNT3 antibodies were able to confirm that binding to the human VEC occurred. However, limitations of specimen resolution restricted the ability to obtain other labeling information. The poor morphology, or distortion, was most probably due to the way the cells were prepared. Centrifugation followed by agarose embedding caused the cells to round up making them difficult to image. However, subsequent high-resolution imaging showed the anti-CNT3 antibody was specifically labeling of microvilli. The specific labeling of the microvilli was confirmed by unbiased PAG10 particle counts on sections from four specimen grids. Specific labeling of VEC plasma membrane was also suggested by the results obtained from two of the grids examined. However, anti-CNT3 labeling on two other grids did not support any specific labeling of the plasma membrane. Although the inconsistent labeling observed on different days may be due to inconsistent antibody dilutions, it is more likely that the differences were due to the heterogeneous expression of the CNT3 protein on the cell membranes. Not only was the label predominantly located on the apical membrane, but it was concentrated in specific regions. While some microvilli strongly labeled, other microvilli in close proximity had reduced labeling.

Labeling of keratin fibers, cell cytoplasm, and other unknown, or unidentified, intracellular structures were at similar levels to the labeling density of support film on all four grids. The location of the CNT3 protein on the microvilli and the plasma membrane is ideal for carrying target molecules into or out of cells. The CNT3 protein must still be in an active state to carry out its function, a condition that is not yet confirmed in freshly shed human VEC.

The initial sampling and quantitative approach applied to evaluating antibody labeling distribution was chosen because it is rapid, unbiased and simple to understand and carry out. Labeled sections are scanned to identify gold particles, the location of which is scored on a panel of predetermined subcellular structures. The collected data can be displayed on a bar chart using either the raw data, or as were presented here, as percentages of the total number of gold particles counted. The results represent observed frequency of labeling over the different subcellular compartments. By labeling two grids in tandem and scoring the labeling patterns independently, we were able to test for correlations between the distributions of gold counts between individual grids of the same labeling experiment. By repeating the labeling on two other grids, we were able to test labeling correlations on grids from different experiments. In all instances, when the Pearson correlation coefficient (r) for each pair was calculated, it was close to 1. The closer the r value is to 1, the better the agreement in the gold label distribution, which supports the initial observation that the anti-CNT3 antibody labeled microvilli on human VEC. Similarly, the χ² tests suggest that the labeling is systematic and not random.

One disadvantage of performing the rapid scanning approach for gold particle counting is its inability to provide reliable estimates of the labeling density over subcellular structures. However, by using cross-lattice overlays on micrographs, it is possible to estimate the amounts of PAG10 particles over the membranes. The PAG10 particles being counted had two locations within the microvilli, either they were close to the outer membrane (≤3 nm) or were between 3 and 5 nm away from the membrane. The counting produced an estimate of 15 PAG10 particles per μm of microvillus membrane (8 PAG10 particles <3 nm from membrane and 7 that were 3–5 nm distance from the microvilli membrane), suggesting a high number of CNT3 molecules present on each structure. The peptide sequence recognized by the CNT3 antibodies is cytoplasmic so labeling of the cytoplasmic regions of the membranes was to be expected. PAG10 particles labeling far from the membrane can be explained if the microvilli are tubular in shape. Calibrated micrographs show the microvilli to have average diameters of approximately 100 nm. The section thickness is only 60 nm, so it is possible that on many sections, the inner parts of the microvilli are also close to the outer membrane. This speculation is supported by the labeling associated with the plasma membrane being mostly <3 nm from the membrane, a result of the plasma membrane profile being perpendicular to the plane of sectioning.

Using a commercial antibody to locate a membrane protein at high-resolution on VEC is seldom performed, most likely because of the limited availability of skills using this approach. By combining the high-resolution imaging with a particulate visualization marker such as a colloidal gold probe, antibody labeling could be quantified and tested for specificity. The antibody used to locate the CNT3 protein did not produce sufficient information at the light microscope level to determine a specific location within VEC. Electron microscopy was able to locate the CNT3 protein on microvilli on the cells, and thus assist in determining possible functional mechanisms for the protein within the cells.

Immunolabeling relies heavily on the quality of reagents being used. The most variable and unreliable of these reagents is the primary antibody that is chosen for use. Other than providing details of the antigen being used to generate the antibody, commercial antibodies are sold with minimal validation. The antibody used in this study has minimal validation studies, which were performed on cells and tissues other than VEC. In addition to identifying the apical microvilli as the primary site for CNT3 expression in VEC, we have also obtained further characterization of these antibodies. A cytoplasmic location for the antigen being recognized by the CNT3 antibodies was confirmed when the antibodies were used to label undisrupted cells. Only cells permeabilized by Triton X-100 would label with the antibodies. The PAG10 used in this study was also confirmed to not bind to the human VEC, indicating the presence of PAG10 on sections being due to specific CNT3 antibody binding. Using serial sections to compare the same region of cell, one labeled with antibody and PAG10 and the other labeled only with PAG10 is a powerful way of confirming the specificity of CNT3 labeling.

**CONCLUSIONS**

The human concentrative nucleoside transporter CNT3 mediates the unidirectional flow of nucleosides, preferring pyrimidine and purine nucleosides. The broad selectivity of this protein gives it the ability to translocate nucleoside-derived anticancer and antiviral drugs. Using high resolution imaging,
the CNT3 transporter was located in the microvilli of VEC from two female volunteers, one Caucasian and one African American. Identifying human transporters on human VEC is relevant when studying normal physiological processes, and when preventing sexually transmitted infections. The transporters play important roles in moving nucleosides and nucleotides, important modulators of nutrient uptake and maintenance of cellular homeostasis, across cell membranes. The specificity of these transporters is not strictly restricted to their respective substrates, so nucleoside analog drugs with significant structural similarity to the physiological substrates have the potential to be recognized and transported by these transporters. Many of the drugs used to treat viral infections are nucleoside and nucleotide analogs, which can interact with the transporter proteins, and be carried across membranes.

### EXPERIMENTAL SECTION

**Cell Collection.** Ethical approval for the involvement of women in this study was consented by the Aspire Institutional Review Board (project #OCIS-02, approved on 07/10/2017). Written informed consent was obtained from all volunteers, and guidelines and policies established by the World Health Organization for good clinical practice and Aspire IRB were followed. Female volunteers were recruited to submit to a self-sampling protocol, where VEC were collected using a menstrual soft cup. VEC were removed from the soft cup by centrifugation, fixed in 4% formalin in 100 mM phosphate buffer and stored at 4 °C until used. The cells used in this validation study were from two randomly selected females: OCIS-02A-037, a healthy Caucasian, and OCIS-02A-02S, an African American. Both volunteers were in their mid 30s.

**Cultured Cells.** Human immortalized VEC (V19 cells) were propagated in an antibiotic-free, keratinocyte serum-free medium (KSFM; Invitrogen, Carlsbad CA) in plastic tissue culture dishes containing sterile, 12 mm diameter round coverslips. The cells were incubated overnight at 37 °C in 5% CO₂ in a humidified incubator. Cells were washed with 1% bovine serum albumin (BSA) in PBS, and incubated with fluorescent secondary antibody and stored at 4 °C until used.

**Immunocytochemistry.** For light microscopy fixed VEC were pelletted and resuspended in low melting point agarose contained in 5% CO₂ in a humidified incubator. Cells were incubated overnight at 37 °C in 5% CO₂ in a humidified incubator. Cells were fixed in 4% formalin in 100 mM phosphate buffer and stored at 4 °C until used.

**Immunolabeling Protocol.** For light microscopy, the collected VEC were washed in PBS containing 0.15% glycine, embedded in 12% gelatin, infiltrated with 2.3 M sucrose and frozen in liquid nitrogen. Freeze substitution (FS) was carried out in 100% methanol containing 1% uranyl acetate following previously described methods. An AFS2 FS device (Leica Microsystems Inc.) warmed the frozen VEC to −80 °C, and then to −60 °C for infiltration with Lowicryl HM20 resin (EMS, Hatfield, PA). The Lowicryl HM20 resin containing the VEC was polymerized with UV light at −50 °C.

60 nm thin sections of VEC were immunolabeled using the same polyclonal antibody against the nucleoside transporter CNT3 as was used for light microscopy, and 10 nm protein A gold, (PAG10, Utrecht University, The Netherlands). Dilutions of immunoreagents with 2% BSA in PBS were made at 1:25 and 1:50 for SLC28A3 and PAG10, respectively. The immunolabeling protocol followed established iterative methods where the grids, section side down, were floated on drops of immunoreagents in a humidity chamber. Immunolabeled sections were contrasted using aqueous uranyl and lead stains. Imaging was performed using a Zeiss EM10C transmission electron microscope operating at 80 kV. For electron microscopy, the collected VEC were washed in PBS containing 0.15% glycine, embedded in 12% gelatin, infiltrated with 2.3 M sucrose and frozen in liquid nitrogen. Freeze substitution (FS) was carried out in 100% methanol containing 1% uranyl acetate following previously described methods. An AFS2 FS device (Leica Microsystems Inc.) warmed the frozen VEC to −80 °C, and then to −60 °C for infiltration with Lowicryl HM20 resin (EMS, Hatfield, PA). The Lowicryl HM20 resin containing the VEC was polymerized with UV light at −50 °C.

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**Quantiﬁcation.** Sections from volunteer OCIS-02A-037 were collected on two separate grids and labeled simultaneously on two different days. The labeling distribution on each of the two labeled grids was compared. Previously described quantification methods to obtain estimates of “pool size” were applied by counting approximately two hundred gold particles per grid for a total of four grids among six cellular compartments (plasma membrane, keratin, cytoplasm, microvilli, unknown intracellular, background). Antigen distributions were obtained from random arrays of micrographs, which were scanned to identify PAG10 particles and assign them to one of the six cellular compartments. Systematic random sampling approaches were applied to eliminate selective bias. To test for reproducible patterns of labeling, the Pearson correlation coefficient (r) was calculated between two sets of two grids. The χ² test was used to test the null hypothesis of uniform labeling across the grids. The χ² test was performed as previously described.

Images of cells labeled with anti-CNT3 from volunteer OCIS-02A-02S were collected from anti-CNT3-labeled regions and from regions of plasma membrane on the same cells where there were no microvilli, and therefore, reduced labeling. The two regions were designated apical (with microvilli) and basolateral (no microvilli). Images were quantified using cross-lattice overlays to enable point counting to be performed. Gold labeling per micron length of membrane was estimated using established protocols. Gold particle labeling on membranes was bilized by 0.1% Triton X-100 in PBS for 10 min, treated with 1% bovine serum albumin (BSA) in PBS, and incubated overnight in anti-CNT-3 antibodies diluted 1:100 in PBS–BSA. The VEC were then washed with PBS, treated with PBS–BSA and incubated with fluorescent secondary antibody also diluted 1:100 in PBS–BSA. The VEC were labeled with DAPI (4',6-diamidino-2-phenylindole) and fluorescent concanavalin A to visualize the cells. The labeled VEC in agarose were mounted on glass slides in Mowiol containing DABCO (1,4-diazabicyclo[2.2.2]octane).

Labelled cells were imaged by confocal laser scanning microscopy (Zeiss LSM 510-Meta, Carl Zeiss AG, Oberkochen, Germany).

For electron microscopy, the collected VEC were washed in PBS containing 0.15% glycine, embedded in 12% gelatin, infiltrated with 2.3 M sucrose and frozen in liquid nitrogen. Freeze substitution (FS) was carried out in 100% methanol containing 1% uranyl acetate following previously described methods. An AFS2 FS device (Leica Microsystems Inc.) warmed the frozen VEC to −80 °C, and then to −60 °C for infiltration with Lowicryl HM20 resin (EMS, Hatfield, PA). The Lowicryl HM20 resin containing the VEC was polymerized with UV light at −50 °C.
was divided between particles closer than 3 nm to the membrane, and particles 3 and 5 nm from the membrane.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02329.

Triton X-100 control. This experiment demonstrates the cytoplasmic location of the CNT3 peptide recognized by the anti-CNT3 antibodies. Cultured V19 cells were preserved in formalin and labeled with anti-CNT3 antibodies followed by Cy3 conjugated secondary antibodies (red). Cells were visualized with FITC-concanavalin A (green) and DAPI (blue). Intact labeled cells, which were not Triton X-100 treated, show weak label with the anti-CNT3 antibodies. Concanavalin A (green) labeled the cell periphery but did not penetrate the cell. Cells treated with Triton X-100 to give access to intracellular components show CNT3 label (red) and intracellular concanavalin A label (green). Scale bar = 20 μm; CNT3 antibodies label apical microvilli on polarized human VEC. Electron micrographs of sections through VEC labeled with anti-CNT3 antibodies and 10 nm protein A gold (PAG10). Most of the PAG10 label was over cytoplasmic regions of the microvilli. The PAG10 labeling is associated with microvilli on one side of the cell (apical microvilli). The membrane on the opposite side of the cell has no microvilli and few PAG10 particles. A different cell has CNT3 labeling over the apical microvilli (arrows). In this image a 100 nm scale bar has been added to illustrate the dimensions of the apical microvilli. Some gold labeling is associated with a keratin bundle (arrowhead). An extracellular bacterium is also present in the image. TheVEC was from an African American female in her mid 30s (OCIS-02A-025). Scale bars = 0.5 μm; and Sequential sections labeled with anti-CNT3 antibodies and PAG10. Two sequential sections through VEC from an African American female in her mid 30s (OCIS-02A-025) labeled with anti-CNT3 antibodies and PAG10. The 60 nm thick sections, 180 nm apart, show the variability of antibody labeling over the same region of one cell. Microvilli on the cell membrane have many gold particles associated with them. Microvilli in the same region of the cell have fewer gold particles. Extracellular bacteria assist in matching the same region on the two sections. Scale bar = 0.5 μm. (PDF)

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Notes

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**ABBREVIATIONS**

BSA bovine serum albumin

CLSM confocal laser scanning microscopy

CNT3 human concentrative nucleoside transporter 3

DABCO 1,4-diazabicyclo[2.2.2]octane

DAPI 4′,6-diamidino-2-phenylindole

IgG immunoglobulin G

PAG10 10 nm sized protein A-gold

PBS phosphate buffered saline

PreP pre-exposure prophylaxis

SLC solute carrier

SCL28A3 gene for the human concentrative nucleoside transporter 3

TEM transmission electron microscopy

VEC vaginal epithelial cell(s)

**REFERENCES**

(1) Mangravite, L. M.; Badagnani, I.; Giacomini, K. M. Nucleoside transporters in the disposition and targeting of nucleoside analogs in the kidney. *Eur. J. Pharmacol.* 2003, 479, 269–281.

(2) Badagnani, I.; Chan, W.; Castro, R. A.; Brett, C. M.; Huang, C. S.; Stryke, D.; Kawamoto, M.; Johns, S. J.; Ferrin, T. E.; Carlson, E. J.; Burchard, E. G.; Giacomini, K. M. Functional analysis of genetic variants in the human concentrative nucleoside transporter 3 (CNT3; SLC28A3). *Pharmacogenomics J.* 2005, 5, 157.

(3) Errasti-Murugarren, E.; Pastor-Anglada, M.; Casado, F. J. Role of CNT3 in the transepithelial flux of nucleosides and nucleoside-derived drugs. *J. Physiol.* 2007, 582, 1249–1260.

(4) Errasti-Murugarren, E.; Molina-Arcas, M.; Casado, F. J.; Pastor-Anglada, M. A splice variant of the SLC28A3 gene encodes a novel human concentrative nucleoside transporter-3 (hCNT3) protein localized in the endoplasmic reticulum. *Faseb. J.* 2009, 23, 172–182.

(5) Al-Ghabeish, M. I. Drug Transporters in the Nasal Epithelia and Their Contribution in Drug Delivery; University of Iowa, 2014.

(6) Berg, T.; Hegelund Myrback, T.; Olson, M.; Seidegärd, J.; Werkström, V.; Zhou, X.-H.; Grunewald, J.; Gustavsson, L.; Nord, M. Gene expression analysis of membrane transporters and drug-metabolizing enzymes in the lung of healthy and COPD subjects. *Pharmacol. Res. Perspect.* 2014, 2, No. e00054.

(7) Godoy, V.; Banares, J. M.; Medina, J. F.; Pastor-Anglada, M. Functional crosstalk between the adenosine transporter CNT3 and purinergic receptors in the biliary epithelia. *J. Hepatol.* 2014, 61, 1337–1343.

(8) Young, J. D.; Yao, S. Y. M.; Baldwin, J. M.; Cass, C. E.; Baldwin, S. A. The human concentrative and equilibrative nucleoside...
transporter families, SLC28 and SLC29. Mol. Aspect. Med. 2013, 34, 529−547.

(9) Lucocq, J. M.; Habermann, A.; Watt, S.; Backer, J. M.; Mayhew, T. M.; Griffiths, G. A rapid method for assessing the redistribution of gold labeling on thin sections. J. Histochem. Cytochem. 2004, 52, 991−1000.

(10) Skrypek, N.; Duchène, B.; Hebbel, M.; Leteurtre, E.; Van Seuningen, I.; Jonckheere, N. The MUC4 mucin mediates gemcitabine resistance of human pancreatic cancer cells via the Concentrative Nucleoside Transporter family. Oncogene 2013, 32, 1714.

(11) Skrypek, N.; Vasseur, R.; Vincent, A.; Duchène, B.; Van Seuningen, I.; Jonckheere, N. The oncogenic receptor ErbB2 modulates gemcitabine and irinotecan/SN-38 chemoresistance of human pancreatic cancer cells via hCNT1 transporter and multidrug-resistance associated protein MRP-2. Oncotarget 2015, 6, 10853.

(12) Zheng, X.; Carstens, J. L.; Kim, J.; Scheible, M.; Kaye, J.; Sugimoto, H.; Wu, C.-C.; LeBlu, V. S.; Kalluri, R. Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. Nature 2015, 527, 525.

(13) Gutierrez, M. M.; Brett, C. M.; Ott, R. J.; Hui, A. C.; Giacomini, K. M. Nucleoside transport in brush border membrane vesicles from human kidney. Biochim. Biophys. Acta, Biomembr. 1992, 1105, 1−9.

(14) Ward, J. L.; Tse, C. M. Nucleoside transport in human colonic epithelial cell lines: evidence for two Na+-independent transport systems in T84 and Caco-2 cells. Biochim. Biophys. Acta, Biomembr. 1999, 1419, 15−22.

(15) Damara, J. L.; Elwi, A. N.; Hunter, C.; Carpenter, P.; Santos, C.; Barron, G. M.; Sun, X.; Baldwin, S. A.; Young, J. D.; Mackey, J. R.; Sawyer, M. B.; Cass, C. E. Localization of broadly selective equilibrative and concentrative nucleoside transporters, hENT1 and hCNT3, in human kidney. Am. J. Physiol.: Renal, Fluid Electrolyte Physiol. 2007, 293, F211−F211.

(16) Griffiths, G.; Lucocq, J. M. Antibodies for immunolabeling by light and electron microscopy: not for the faint hearted. Histochem. Cell Biol. 2014, 142, 347−360.

(17) Couchman, J. R. Commercial antibodies: the good, bad, and really ugly. J. Histochem. Cytochem. 2009, 57, 7−8.

(18) Bordeau, J.; Welsh, A. W.; Agarwal, S.; Killiam, E.; Baquero, M. T.; Hanna, J. A.; Anagnostou, V. K.; Rimm, D. L. Antibody validation. Biotechniques 2010, 48, 197−209.

(19) Pastor-Anglada, M.; Pérez-Torras, S. Nucleoside transporter proteins as biomarkers of drug responsiveness and drug targets. Front. Pharmacol. 2015, 6, 13.

(20) Boskey, E. R.; Moench, T. R.; Hees, P. S.; Cone, R. A. A self-sampling method to obtain large volumes of undiluted cervicovaginal secretions. Sex. Transm. Dis. 2003, 30, 107−109.

(21) Pyles, R. B.; Vincent, K. L.; Baum, M. M.; Elsom, B.; Miller, A. L.; Maxwell, C.; Eaves-Pyles, T. D.; Li, G.; Popov, V. L.; Nusbaum, R. J.; Ferguson, M. R. Cultivated vaginal microbe community structure and antiretroviral efficacy in colonized epithelial multilayer cultures. PLoS One 2014, 9, e93419.

(22) Rose, W. A.; II; McGowin, C. L.; Spagnuolo, R. A.; Eaves-Pyles, T. D.; Popov, V. L.; Pyles, R. B. Commensal bacteria modulate innate immune responses of vaginal epithelial cell multilayer cultures. PLoS One 2012, 7, No. e32728.

(23) https://www.uniprot.org/uniprot/Q9HAS3 (accessed August 5th 2020).

(24) Johnson, Z. L.; Cheong, C.-G.; Lee, S.-Y. Crystal structure of a concentrative nucleoside transporter from Vibrio cholerae at 2.4 Å. Nature 2012, 483, 489−493.

(25) Johnson, Z. L.; Lee, J.-H.; Lee, K.; Lee, M.; Kwon, D.-Y.; Hong, J.; Lee, S.-Y. Structural basis of nucleoside and nucleotide drug selectivity by concentrative nucleoside transporters. eLife 2014, 3, No. e03604.

(26) Latek, D. Rosetta Broker for membrane protein structure prediction: concentrative nucleoside transporter 3 and corticotropin-releasing factor receptor 1 test cases. BMC Struct. Biol. 2017, 17, 8.

(27) Webster, P.; Webster, A. Cryosectioning Fixed and Cryoprotected Biological Material for Immunocytochemistry. Electron Microscopy; Springer: 2014; Vol. 1117, pp 273−313.

(28) Kapusciniski, J. DAPI: a DNA-specific fluorescent probe. Biotech. Histochem. 1995, 70, 220−233.

(29) Lennette, D. A. An improved mounting medium for immunofluorescence microscopy. Am. J. Clin. Pathol. 1978, 69, 647−648.

(30) Johnson, G. D.; Davidson, R. S.; McNamee, K. C.; Russell, G.; Goodwin, D.; Holborow, E. J. Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. J. Immunol. Methods 1982, 55, 231−242.

(31) Ono, M.; Murakami, T.; Kudo, A.; Ishikii, M.; Sawada, H.; Segawa, A. Quantitative comparison of anti-fading mounting media for confocal laser scanning microscopy. J. Histochem. Cytochem. 2001, 49, 305−311.

(32) van Genderen, I. L.; Van Meer, G.; Slot, J. W.; Geuze, H. J.; Voorhout, W. F. Subcellular localization of Forssman glycolipid in epithelial MDCK cells by immuno-electronmicroscopy after freeze-substitution. J. Cell Biol. 1991, 115, 1009−1019.

(33) Gundersen, H. J. G.; Jensen, E. B. V.; Kieu, K.; Nielsen, J. The efficiency of systematic sampling in stereology— reconsidered. J. Microsc. 1999, 193, 199−211.

(34) Griffiths, G. Fine Structure Immunocytochemistry; Springer Science & Business Media, 2012.