Primary Human Proximal Tubule Model for the Study of Transepithelial Transport

Caitlin Brown[1], Yuko Kosaka[2], Marc Evanchik[2], Scott Heyward[1], and Ji Young Lee[1]

(1) BioreclamationIVT (Baltimore, MD) (2) 3-V Biosciences (Menlo Park, CA)

ABSTRACT

Renal elimination is a major clearance route for many drugs and their metabolites. This includes active tubular secretion, which takes place primarily in the proximal tubule (PT) cells, and is mediated by uptake and efflux transporters. Currently, most *in vitro* kidney transporter data are obtained from renal slices, isolated tubule fragments, or animal-derived cell lines that can exhibit differing transport characteristics or altered transporter behavior compared to human. Primary cells cultured on transwells have been shown to be an effective *in vitro* model and may represent a more physiologically relevant system.[1,2] To that end, freshly isolated primary kidney cells were plated onto semipermeable membrane filters and assessed for monolayer integrity and transporter functionality. A gamma glutamyltransferase (GGT) assay was used to determine percentage of PT cells with respect to distal tubule (DT) cells. An approximate 4:1 ratio of PT to DT cells has been determined to be optimal for appropriate tight junction formation.[1] In addition, transepithelial electrical resistance (TEER) measurements and mannitol permeability verified monolayer integrity. Prototypical substrates, para-aminohippuric acid (PAH) and 1-methyl-4-phenylpyridinium (MPP$^+$), were used to evaluate expression of the key transporter pathways OATs to MRP2/4 and OCT2 to MATE1, respectively. A flux ratio of secretory to absorptive (B→A/A→B) greater than 1.5 was defined as significant.[1] Cultured cells showed secretory flux for both PAH and MPP$^+$. Transporter function for PAH diminished or was abolished over time. Shipment of these transwell cultures on agarose media caused a drop in TEER values and a loss of net flux after transfer to liquid media. However, after some days in culture, transepithelial transport was re-established as measured by MPP$^+$ movement. Thus, a primary kidney cell model has been shown to present appropriate transporter functioning and retention of these transporters upon shipment in an agarose system.

INTRODUCTION

The kidney is the principle excretory organ in the human body. Within the kidney, nephron clearance involves glomerular filtration, active tubular secretion, and tubular reabsorption. Active tubular secretion is mainly mediated by PT cells. PT cells take up xenobiotics using uptake transporters located on the basolateral membrane (blood side) followed by secretion across the apical membrane (urine/lumen side) into the urine.[3] Elucidating transport pathways allows better understanding of drug disposition. To best mimic an *in vivo* system, one can study transporters using freshly isolated human primary PT cells plated on semipermeable filters known as transwells. When plated on transwell plates, cells form a polarized monolayer, allowing media exposure to both the basolateral and apical membranes. Not only can uptake and efflux transport can be studied, but also drug-drug interactions can be evaluated. With the use of prototypical substrates, bidirectional transepithelial flux studies were tested to assess functionality of essential uptake transporters, such as OAT1/3 and OCT2, as well as essential efflux transporters, such as MRP2/4 and MATE1/2.[4,5] This is an effective and efficient *in vitro* model that allows retention of an intact, healthy monolayer as well as expression of key transporters involved in drug disposition.

MATERIALS & METHODS

Reagents

[^{3}H]Para-aminohippuric acid (PAH) was purchased from PerkinElmer (Boston, MA). [^{3}H]Mannitol was purchased from American Radiochemicals (St. Louis, MO). Methyl-4-phenylpyridinium (MPP$^+$) was purchased from Sigma. Worthington Type 2 collagenase was purchased from Worthington Biochemicals (Lakewood, NJ). Gamma glutamyltransferase (GGT), BioreclamationIVT Proximal Tubule (PT) media, IVT Hanks Balanced Salt Solution (HBSS) Perfusion Buffer, and IVT HBSS Digestion Buffer were made in-house at Celsis In Vitro Technologies.

Cell Isolation

Human renal PT cells were isolated from an intact male human kidney based on the procedure outlined in Brown et al., 2008 with minor modifications. Briefly, the kidney was perfused aseptically for about 10 minutes with BioreclamationIVT HBSS Perfusion Buffer until flushed. The kidney was decapsulated and cortex was dissected into small pieces and digested with Worthington Type 2 collagenase at a concentration of 0.67 mg/mL in IVT HBSS Digestion Buffer. Cortex slices were digested overnight at 4ºC on a shaker. Cells were filtered through 120 µm mesh and washed with BioreclamationIVT PT media. The cell suspension was layered carefully on discontinuous Percoll gradient layers of 1.04 and 1.07 mg/mL. Cells were centrifuged at 1942xg for 25 minutes. Cells were harvested from the border of the two Percoll layers and contained PT and distal tubular/collecting duct (DT/CD) cells. Cells were washed twice with PT media, counted using a hemocytometer, and adjusted to a concentration of 33,000 cells/mL. Cells were plated on 12 well polycarbonate and polyester transwell plates.

Cell Monolayer Analysis

Transepithelial electrical resistance (TEER) readings were measured every day starting on day 3 to evaluate the cell monolayer. Along with TEERs, mannitol flux assay was performed to also evaluate the cell monolayer's tight junction. A GGT assay was performed for every isolation on polyester plates for visual inspection to determine the cell population.

Flux Experiments

Bidirectional flux assays were done on 12 well polycarbonate transwell plates. Prototypical substrates, [^{3}H]PAH (1 µCi/mL), MPP$^+$ (1µM), and [^{3}H]mannitol (0.1µCi/mL) were added to independent basolateral and apical compartments. Samples were collected from the opposite compartment and read on a Beckman liquid scintillation counter for transport measurements.

RESULTS

Gamma Glutamyltransferase Assay

A GGT assay was used to determine percentage of PT cells with respect to DT cells. The colorimetric assay dyes the brush border membrane found uniquely in PT cells a bright red color that can be detected visually. The assay was done on day 7 for every isolation for comparison.

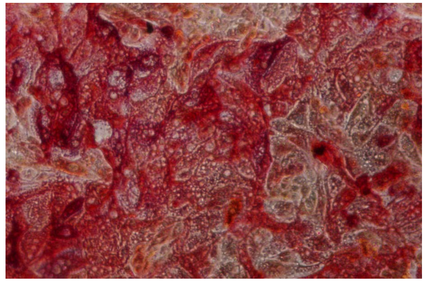

Figure 1: Representative micrograph of a cell monolayer stained using the GGT assay

Mannitol

Radiolabeled [^{3}H]mannitol (0.1µCi/mL) flux studies were done to determine the amount of paracellular movement between the two transwell compartments, providing tight junction analysis. When tight junctions are established within the monolayer, the mannitol flux is reduced due to the substrate only crossing the monolayer in a paracellular fashion. Ideally, net flux should not be statistically different from zero. In figure 2, mannitol net flux values were graphed against the associated TEER values from the same time point. When TEERs reached about 170Ω, mannitol permeability was drastically reduced. Thus, compound flux after 170Ω is due to transcellular transport across the cell membrane.

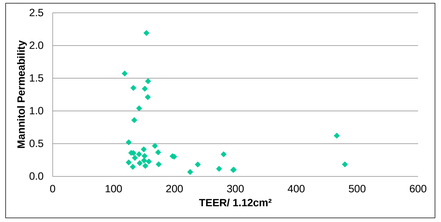

Figure 2: Mannitol permeability compared with individual TEER values from six kidney isolations.

TEER Values

Transwell plates were placed in an agarose gel and shipped overnight to 3-V Biosciences. Upon arrival, cells were transferred to a fresh plate containing 37^{0}C PT media. TEERs were read starting from the next day. External TEER values were compared to in-house TEER values.

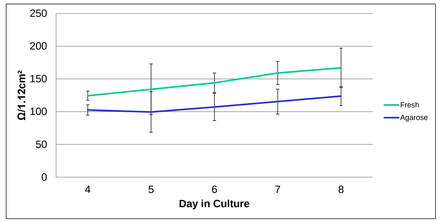

Figure 3: TEER values of fresh renal PT plates in-house compared to TEER values of shipped agarose plates. Results are an average of four kidneys.

PAH

Radiolabeled [^{3}H]PAH (10µM), a prototypical substrate, was tested in-house to assess OAT1/3→MRP2/4 pathway. Due to donor-to-donor variation, secretory flux varied days in culture. A flux ratio of secretory to absorptive (B→A/A→B) greater than 1.5 was defined as significant and was seen every isolation on day 6 and earlier. Furthermore, in every isolation expression of transport diminished over time.

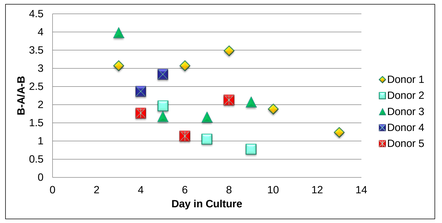

Figure 4: PAH fold flux after days in culture

MPP$^+$

A prototypical organic cationic substrate, MPP$^+$, was used to assess the expression of the OCT2→MATE1/2 pathway on monolayers after shipment. Plates were shipped in agarose media to 3-V Biosciences in Menlo Park, CA. MPP$^+$ (1µM) flux was measured by LC/MS/MS. Fold flux greater than 1.5 (B→A/A→B) was seen for three isolations.

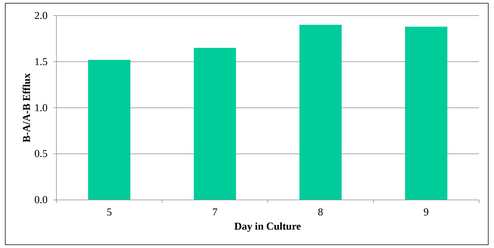

Figure 5: MPP flux after shipment days in culture.

CONCLUSIONS

- **GGT staining confirmed the appropriate percentages of PT to DT cells in culture.**
- **The comparison between TEER values and mannitol flux showed that when TEER values were greater than about 170Ω, mannitol permeability greatly diminished. Compound transport after this time is due to transcellular transport.**
- **Bidirectional transepithelial flux assays showed expression of OAT1/3 and MRP2/4 pathway with use of the substrate PAH. Although day of flux varied from donor to donor, positive secretion of ≥1.5 B-A/A-B flux was seen in every isolation before day 6. Transporter expression diminished over time, proving assays should be run before day 6.**
- **TEER values before and after shipment were similar, showing retention of intact monolayers after shipment.**
- **Flux of ≥1.5 B-A/A-B for MPP$^+$ showed retention of OCT2 and MATE1/2 transport expression after shipment.**

REFERENCES

1. Brown CDA *et al.* Toxicol Appl Pharm 223: 428-438 (2008).
2. Lash LH *et al.* Toxicology 228: 200-218 (2006).
3. Ray AS *et al.* Antimicrob Agents Chemother 50: 3297-3304 (2006).
4. Schwabedissen HEM *et al.* Am J Physiol Renal Physiol 298: 997-1005 (2010).
5. Terada T *et al.* Biochem Pharmacol 75: 1689-1696 (2008).