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Albumin handling by renal tubular epithelial cells in a microfluidic bioreactor

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Abstract

Epithelial cells in the proximal tubule of the kidney reclaim and metabolize protein from the glomerular filtrate. Proteinuria, an overabundance of protein in the urine, affects tubular cell function and is a major factor in the progression of chronic kidney disease. By developing experimental systems to study tubular protein handling in a setting that simulates some of the environmental conditions of the kidney tubule *in vivo*, we can better understand how microenvironmental conditions affect cellular protein handling to determine if these conditions are relevant in disease. To this end, we used two *in vitro* microfluidic models to evaluate albumin handling by renal proximal tubule cells. For the first system, cells were grown in a microfluidic channel and perfused with physiological levels of shear stress to evaluate the role of mechanical stress on protein uptake. In the second system, a porous membrane was used to separate an apical and basolateral compartment to evaluate the fate of protein following cellular metabolism. Opossum kidney (OK) epithelial cells were exposed to fluorescently labeled albumin, and cellular uptake was determined by measuring the fluorescence of cell lysates. Confocal fluorescence microscopy was used to compare uptake in cells grown under flow and static conditions. Albumin processed by the cells was examined by size exclusion chromatography (SEC) and SDS-PAGE. Results showed that cellular uptake and/or degradation was significantly increased in cells exposed to flow compared to static conditions. This was confirmed by confocal microscopy. Size exclusion chromatography and SDS-PAGE showed that albumin was broken down into small molecular weight fragments and excreted by the cells. No trace of intact albumin was detectable by either SEC or SDS-PAGE. These results indicate that fluid shear stress are important factors mediating cellular protein handling and microfluidic *in vitro* models provide a novel tool to investigate these processes.

Keywords

endocytosis; proximal tubule; microfluidic; shear stress; epithelial cells

Introduction

In a properly functioning kidney, blood is filtered at the glomerulus where water and low molecular weight solutes are freely filtered while cells and larger molecular weight solutes are retained in the blood. The filtrate passes into the tubule where a majority of the salt and water are reabsorbed while toxins remain in the urine. In addition to reabsorption of salt and

water, epithelial cells in the proximal tubule also metabolize albumin and other proteins that cross the glomerular filter. It is well established that some albumin crosses the glomerular filter, but in health, almost none of the intact protein remains in the urine. The actual amount of albumin that passes through the glomerulus is hotly debated and remains an active area of research. Estimates of glomerular albumin sieving coefficient, the ratio of albumin concentration in the filtrate to that in the blood, vary widely from 6×10^{-4} (Tojo and Endou, 1992) to 0.034 (Russo et al., 2007) in healthy rats. As a consequence, it remains unclear what role tubular protein uptake plays in maintaining sub-nephrotic levels of protein in the urine. If significant amounts of albumin pass into the tubular filtrate, this may provide alternative targets for therapeutic intervention for treatment of proteinuric renal disease.

Receptor mediated endocytosis by megalin and cubilin has been established as one mechanism of proximal tubule cell uptake (Zhai et al., 2000). Figure 1 shows a schematic diagram of receptor mediated cellular uptake, processing, and excretion of albumin. Albumin is bound at the apical cell membrane where it is internalized and eventually transported to the lysosome for degradation. The receptor is then recycled back to the membrane. The degradation products are excreted back into the perfusion medium through the apical membrane and/or across the basolateral membrane. Receptor mediated binding, uptake, trafficking, and lysosomal degradation of albumin have been studied in cell culture models, especially in opossum kidney (OK) cells (Schwegler et al., 1991; Gekle et al., 1995a,b; Gekle et al. 1996;). However, there is a growing body of evidence that this is not the only pathway for albumin reclamation (Russo et al., 2007; Eppel et al., 1999; Sarav et al., 2009). Furthermore, the size and destination of the degraded fragments remains unclear. Initial studies suggested that the fragments were completely excreted across the basolateral cell membrane and into the blood for recycling (Park and Maack, 1984). Additional work has since shown that significant amounts of the low molecular weight fragments (<10 kD) are excreted across the apical cell membrane and into the urine (Clavant et al., 2003). This would imply that traditional estimates of urinary protein loss may be significantly underestimated since traditional protein assays may not detect the smaller molecular weight fragments (Eppel et al., 2000; Greive et al., 2001). Still others have observed albumin fragments excreted into both the apical and basolateral space in cell culture models (Gudehithlu et al., 2004).

Tubular protein handling has significant implications in disease. In individuals with chronic kidney diseases such as diabetic nephropathy, which is characterized by sustained proteinuria, not only is the capacity for tubular protein reabsorption exceeded, but the capacity of the tubule cells to process albumin is diminished (Tojo et al., 2001; Osicka et al., 2000). Other diseases that diminish the capacity for tubular reabsorption may directly cause proteinuria independently of glomerular damage. This has been hypothesized in the case of proteinuria associated with Fanconi syndrome (Norden et al., 2002) and Dent's disease (Wang et al., 2000). Regardless of whether the cause of proteinuria is increased glomerular sieving or a defect in tubular protein handling, the long term consequence is often tubular inflammation and fibrosis resulting in further disease progression and eventually end stage renal disease (ESRD) (Birn and Christensen, 2006; Pollock and Poronnik, 2007). Additional understanding of the mechanisms governing tubular protein uptake and modification of uptake capacity may guide therapeutic interventions to slow the progression of chronic kidney disease.

Microfluidic devices provide a powerful tool for replicating cellular microenvironment found in the kidney tubule to investigate protein trafficking in proximal tubule cells. Proximal tubules are essentially microscale circular ducts with luminal diameters of approximately 20–25 μm and flow rates in of approximately 10–20 nl/min (Maunsbach et al., 1987; Chou and Marsh, 1987) under normal physiological conditions. Microfluidic devices provide a

convenient means of replicating the architecture and mechanical conditions found in the proximal tubule to investigate the role of these factors in regulating cellular function. Mechanical forces, particularly shear stress at the apical cell surface, have been clearly implicated as significant regulatory factors in membrane trafficking in a host of cells and tissues (Apodaca, 2002), but few cell culture models replicate the physical and mechanical conditioned experienced by cells *in vivo*. We used two microfluidic models, a single chamber and multi-chamber microfluidic bioreactor (Ferrell et al., 2010), to investigate the role of fluid flow in altering tubular protein uptake and to evaluate the fate of metabolized albumin by proximal tubule epithelial cells. This experimental model may provide a useful organotypic cell culture model to further study the role of protein metabolism in altering proximal tubule cell function and its role in progression of chronic kidney disease.

Methods

Microfluidics Devices

Two microfluidic systems were used in this study. The first was a simple single chamber microfluidic device bonded to a glass substrate. This will be referred to as the single chamber bioreactor. Since cell growth on porous substrates is known to alter the level of differentiation of polarized epithelial cells (Lever, 1986; Orosz et al., 2004), the single chamber systems was used to assess the influence of flow on uptake of albumin without the additional influence of growing cells on a porous substrate. The second system was a multi-chamber bioreactor consisting of apical microchannels for perfusion and a basolateral compartment, with a porous membrane as a support for cell growth and pathway for apical to basolateral transport. Details on the device fabrication and characterization of cell growth in the bioreactor can be found in (Ferrell et al., 2010). A cross-sectional schematic of the single chamber bioreactor is shown in Figure 2(a). A schematic and scanning electron micrograph of the multi-chamber bioreactor is shown in Figure 2(b,c), respectively. A polycarbonate membrane separates the apical and basolateral compartment. Cells are grown on the membrane in the apical compartment.

Cell culture

Opossum kidney (OK) cells (ATCC, CRL-1840) were used as a model cell line because they are a well established renal epithelial cell line that internalizes albumin predominantly via receptor-mediated endocytosis (Schwegler et al., 1991; Zhai et al., 2000). OK cells were grown in minimum essential medium (MEM - Invitrogen, 0820234DJ) with 2 mM L-glutamine (Invitrogen, 25030-164), 10% fetal bovine serum (Invitrogen, 10091-148), and 1% antibiotic/antimycotic solution (Invitrogen, 15240-062). Culture medium was changed three times per week. Cells were used at passage 45–51.

Albumin Uptake

Cells were grown to confluence plus three days in the single chamber microfluidic systems. Static controls were grown in 6 well cell culture dishes. Cells were serum starved for 24 h prior to albumin exposure. Cells were then exposed to MEM containing 50 $\mu\text{g/ml}$ FITC-albumin (Sigma, A9771) for 3 hours at a flow rate equivalent to 1 dyn/cm^2 shear stress at the apical surface of the cells. The 3 hour time period was chosen to provide time for sufficient cellular uptake of FITC-albumin. In addition to internalized intact protein, degradation of the protein is likely a contributor to the measured fluorescence. Fluorescence intensity increases as the protein is degraded, likely by exposing FITC that is quenched in the non-degraded protein conformation (Ohshita and Katunuma, 1992). The shear stress value was chosen as an approximation of shear stress conditions at the walls of tubules *in vivo* based on structural and flow rate data from rat tubules (Maunsbach et al., 1987; Chou and Marsh, 1987). After exposure, cells were washed with cold PBS. A sample of the cell

suspension was collected prior to cell lysis and cells were counted and fluorescence of the lysates was normalized to cell number.

Albumin Characterization

Uptake was characterized by fluorescence microscopy and by analyzing the fluorescence of cell lysates. Imaging was performed using confocal microscopy (Leica, DMR). Cell lysate fluorescence was measured using a fluorescence plate reader (PerSeptive Biosystems, Cytofluor II). Cells were trypsinized, collected, pelleted by centrifugation, and lysed in 1% Triton X-100 in 100 mM HEPES buffered saline at pH 7.4. Lysates were analyzed on a fluorescence plate reader with excitation at 485 nm and emission at 530 nm.

Size exclusion chromatography (SEC) was used to separate and detect intact and degraded albumin. Cells were grown in both the single chamber and multi-chamber bioreactors and serum starved as described previously. Cells were exposed to FITC-albumin continuously for 24 hours. Following exposure, albumin containing medium was replaced with protein free medium for an additional 24 hours. This provided sufficient time for the cells to excrete the processed albumin into the medium in measurable quantities. This is similar to the time scale (18 h) used by Gudehithlu *et al.* (2004). This medium containing processed albumin will be referred to as conditioned medium. Conditioned medium and stock solution with intact FITC-albumin were analyzed by fluorescence detection (495 nm ex. / 520 nm em.) after SEC (Agilent 1200 series). Separation was performed on a TSKgel G3000SWxl column (7.8 mm ID×30 cm, 5 μ M, TOSOH Bioscience, LLC, Japan) at 30 °C, eluting 0.5 mL/min, with a 20 μ L injection volume. Retention time was normalized to an internal standard (100 μ g/mL para-aminobenzoic acid, PABA), run at the beginning of chromatography each day to mitigate column variability. Fluorescence signal was plotted as a function of elution time normalized to PABA elution time. Areas under the curves (AUC) were determined using ChemStation data analysis software (Rev. B.03.02[341] – Agilent Technologies).

Conditioned medium from the single chamber bioreactors was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Conditioned medium was mixed with sample buffer and run on a 10–20% gradient Tris-HCl gel with a pre-stained broad range molecular weight marker (7.1–209 kD) (Bio-Rad, 161-0318). Gels were washed and imaged with both visible light to identify the markers and fluorescence to identify the FITC-albumin. Fluorescence imaging was performed using a STORM 840 Imager (GE Healthcare) with 480 nm excitation and 530 nm emission. Optical and fluorescence images were aligned using image analysis software to create composite images.

Results and Discussion

To evaluate the effect of fluid flow of cellular albumin uptake, cells were perfused with FITC-albumin and uptake was compared with that of cells grown under static conditions using confocal fluorescence microscopy and by measuring fluorescence of cell lysates with a plate reader. Uptake of FITC-albumin under flow resulted in a significant increase in cellular fluorescence compared with cells grown under static conditions. Fluorescence of cell lysates following FITC-albumin exposure was quantified using a plate reader. Mean fluorescence per 10^6 cells is plotted in Figure 3a. The mean fluorescence increased almost 3-fold in the cells exposed to flow. Difference in the mean fluorescence was statistically significant ($p < 0.05$), based on a Student's t-test. For flow, $n=28$ samples and for static $n=33$. The use of FITC as a fluorescent probe raises several issues with regard to direct quantification protein concentration. FITC fluorescence is pH sensitive and undergoes significant photobleaching. Degradation of FITC-albumin results in increased fluorescence due to excitation of FITC that is quenched in the native protein conformation. In addition,

breakdown products of albumin metabolism may not all be labeled with a FITC molecule, and therefore would not be detected using fluorescence based-analysis. Therefore, all fluorescence data is normalized to control samples at the same excitation conditions and buffered to neutral pH. A single slice from confocal imaging of static uptake is shown in Figure 3b. This albumin uptake pattern is similar as seen in other studies (Zhai et al., 2000; Gudehithlu et al., 2004) with diffuse cellular staining and additional higher intensity localized fluorescence. Presumably, the diffuse staining indicates processed albumin fragments, while the punctate fluorescence indicates FITC-albumin localized in the endocytic vesicles and lysosomes (Gekle, 2005). Under flow (Figure 3c), a significant increase in diffuse staining can be seen with respect to both distribution and intensity. Localized areas of fluorescence can also be seen in the micrograph.

The increase in cellular uptake/degradation could be a result of a variety of factors. Fluid flow is known to induce significant changes in cell phenotype with respect to cell height (Jang and Suh, 2010), cytoskeletal arrangement (Essig et al., 2001; Duan et al., 2008), and protein expression (Kaysen et al., 1999). These phenotypic changes may affect availability and density of receptors at the apical membrane, increase apical membrane surface area, or induce changes in cellular trafficking and breakdown of proteins. Fluid flow has been shown to induce changes in protein activity, protein expression, and membrane localization. For example, fluid flow effects NHE3 (sodium/hydrogen exchanger isoform 3) activity (Du et al., 2006) as well as expression and trafficking to the apical membrane of proximal tubule cells (Duan et al., 2010). NHE3 plays a role in regulation of protein uptake in cells (Gekle et al., 1999). This link could establish a relationship between fluid flow and regulation of protein uptake and processing, although understanding these complex processes will require significant further investigation.

Size exclusion chromatography was used to assess whether any intact albumin was released from the cells following uptake and to estimate the size of albumin fragments. The single chamber bioreactor was used to determine if albumin fragments were released from the apical side of the cells in the absence of a basolateral compartment, and the multi-chamber bioreactor was used to evaluate the presence of fragments in both the apical and basolateral compartments. Results indicated no measurable intact albumin was released into the apical compartment in the single chamber bioreactor, and no intact albumin peak was detected in either the apical or basolateral compartments in the multi-chamber bioreactor (Figure 4a). The chromatographs for all three conditions tested are overlaid in Figure 4a, but overlap of the graphs makes it difficult to differentiate the separate curves. The primary peaks corresponding to degraded albumin are indicated with arrows in Figure 4b. Based on the properties of the SEC column, it can be concluded that the fragments are smaller than 10 kD, the exclusion limit of the separation column, but no more precise estimation of size can be determined from the SEC data. In the absence of a basolateral compartment, OK cells excreted degraded albumin into the apical compartment. In the multi-chamber bioreactor, fragments were detected in both the apical and basolateral compartments. A summary of the mean fluorescence (area under the fluorescence curve) for intact FITC-albumin and the primary degradation peak in the multi-chamber bioreactor are summarized in Table 1. No intact FITC-albumin was detected in the conditioned medium in either the apical or basolateral chamber (n=4 for each case). This suggests all of the protein accumulated in the cell during the 24 hours of exposure is broken down prior to excretion from the cell. This does not necessarily preclude the possibility of transcellular passage of intact protein, as this process likely occurs over a much shorter time period of seconds rather than hours, and may not be captured in these experiments (Comper et al., 2008). The increase in the AUC values for the apical conditioned medium compared to the basolateral medium may suggest that more albumin fragments are excreted from the apical membrane, especially given that the volume of the apical reservoir is significantly larger than the volume of the basolateral

chamber. However, quantification of the ratio of fragments excreted into each compartment is complicated by several factors including the use of FITC as a fluorescent tag, the difference in apical and basolateral volume, the possibility of slight dilution of the basolateral medium during collection, and the possibility of paracellular passage of small molecular weight species between compartments.

SDS-PAGE was used to get a better estimate of the molecular weight range for the fragments. Figure 5 shows the SDS-PAGE of condition medium after exposure to protein-free medium for 24 hours. Results indicated the fragments were smaller than the minimum band in the marker, suggesting the fragments are smaller than 7 kD. This is in agreement with the conclusions of Greive *et al.* (2001), who found significant amounts of small molecular weight fragments (< 10 kD) are excreted into the urine. Gudehithlu *et al.* (2004) evaluated albumin fragments excreted by HK-2 cells. While they too saw albumin fragments in conditioned medium, their analysis revealed fragments much larger (> 45 kD) than those in this study. Differences may be a result of differences in exposure and processing times or differences in the cell line studied.

Conclusion

Two microfluidic systems, one with a basolateral compartment and one without, were used to evaluate albumin degradation by renal proximal tubule cells under shear stress conditions that simulate the *in vivo* environment in the proximal tubule. Confocal fluorescence microscopy and cellular fluorescence analysis showed physiological levels of apical shear stress significantly increased uptake and/or degradation capacity. SEC and SDS-PAGE suggest albumin is degraded into small fragments with sizes < 7 kD and excreted into both apical and basolateral compartments. This tool may be valuable for further study of the role of protein processing by tubule epithelial cells in the progression of chronic kidney disease.

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Figure 1. Schematic of albumin processing by OK cells. Albumin is taken up by the cell via receptor mediated endocytosis, followed by trafficking, breakdown, and excretion of degraded fragments.

Figure 2.

(a) Cross-sectional schematics of (a) the single chamber bioreactor and (b) the multi-chamber bioreactor. (c) Cross-sectional scanning electron micrograph of the multi-chamber bioreactor.

Figure 3.

(a) Fluorescence (normalized to static fluorescence) of cell lysates for cells grown under static and flow conditions showing a significant increase in fluorescence signal when cells are grown under flow as compared to static condition (* $p < 0.05$). Confocal fluorescence micrographs of OK cells following FITC-BSA exposure for (b) cells grown under static condition and (c) grown with fluid flow equivalent to 1 dyn/cm² of shear stress. Areas of punctate, likely lysosomal fluorescence, are indicated with arrows in (b) and (c).

Figure 4.

(a) Chromatographs of the perfusion solution (50 $\mu\text{g/ml}$ FITC-BSA) and conditioned medium from the apical compartment of the single chamber bioreactor and the apical and basolateral compartment of the multi-chamber bioreactor. No intact albumin is detectable in any of the conditioned medium. (b) Chromatographs of degraded albumin in the apical medium of the flat plat bioreactor and the apical and basolateral compartments of the multi-chamber bioreactor.

Figure 5. SDS-PAGE gel of the perfusion solution (50 µg/ml) and conditioned medium from the apical chamber of the single chamber bioreactor showing fragments smaller than the lowest molecular weight band in the marker, indicating fragments < 7.1 kDa.

Table 1

Peak areas for intact and degraded albumin in conditioned medium after 24 hours of cell exposure. No intact albumin was found in any case. ND: none detected

	Intact Peak Area (n=4)	Degraded Peak Area (n=4) (±SE)
Multi-chamber Bioreactor-Apical	ND	66.6 ± 8.9
Multi-chamber Bioreactor-Basolateral	ND	31.7 ± 6.7