Lack of APOL1 in proximal tubules of normal human kidneys and proteinuric APOL1 transgenic mouse kidneys

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Abstract

The mechanism of pathogenesis associated with APOL1 polymorphisms and risk for non-diabetic chronic kidney disease (CKD) is not fully understood. Prior studies have minimized a causal role for the circulating APOL1 protein, thus efforts to understand kidney pathogenesis have focused on APOL1 expressed in renal cells. Of the kidney cells reported to express APOL1, the proximal tubule expression patterns are inconsistent in published reports, and whether APOL1 is synthesized by the proximal tubule or possibly APOL1 protein in the blood is filtered and reabsorbed by the proximal tubule remains unclear. Using both protein and mRNA in situ methods, the kidney expression pattern of APOL1 was examined in normal human and proteinuric APOL1 transgenic mice with and without proteinuria. APOL1 protein and mRNA was detected in podocytes and endothelial cells, but not in tubular epithelia. In the setting of proteinuria, plasma APOL1 protein did not appear to be filtered or reabsorbed by the proximal tubule. A side-by-side examination of commercial antibodies used in prior studies suggest the original reports of APOL1 in proximal tubules likely reflects antibody non-specificity. As such, APOL1 expression in podocytes and endothelia should remain the focus for mechanistic studies in the APOL1-mediated kidney diseases.

Introduction

Polymorphisms in the APOL1 gene contribute significant risk for several forms of non-diabetic chronic kidney disease (CKD) [1–3]. This risk arises from a combination of recessive...
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APOL1 and proximal tubules

inheritance of variant APOL1 alleles plus exposure to an environmental stressor. The pathogenic function of the APOL1 variants and how they interact with the environmental stressor to cause CKD are not fully understood. Although APOL1 is constitutively present in the circulation, prior studies have minimized a causal role for the circulating APOL1 protein [4–7], and efforts to understand kidney pathogenesis have focused on APOL1 expressed in renal cells. The APOL1 kidney expression pattern remains unclear with published discrepancies between immunohistochemistry and mRNA in situ hybridization results, most notably the abundant APOL1 protein observed in the proximal tubule epithelium [8–10]. Since APOL1 is abundant in blood, it is unclear if APOL1 is filtered, especially in the setting of proteinuria, which could result in APOL1 protein reabsorption by the proximal tubule. Appearance of APOL1 in the proximal tubule, either by gene expression or reabsorption from filtrate, would indicate a potentially important role of the proximal tubule in APOL1-associated CKD pathogenesis.

APOL1 in circulation is bound to a 500 kDa HDL3 particle, known as trypanolytic factor 1, a 1000 kDa lipid-poor IgM complex, known as trypanolytic factor 2, and possibly other lipid-poor, high molecular complexes associated with complement factors [7, 11–13]. The proteins produced by the two CKD-associated APOL1 variant alleles, G1 and G2, bind the high molecular weight trypanolytic factors similar to the common allele G0 [14]. Although the APOL1 protein (42.5 kDa) is small enough to pass the glomerular filtration barrier size restriction limit, it is not known to circulate independent of these high molecular weight complexes [15]. However, lipoproteins and other components of HDLs can be filtered [16], and in the setting of proteinuria, larger molecular weight proteins normally restricted by the filtration barrier may appear in filtrate. It is unclear whether APOL1 or APOL1-containing complexes may be filtered in the setting of proteinuria.

To resolve these issues, we examined both APOL1 gene and protein expression in human kidney tissue and kidneys from humanized transgenic mouse models that recreate native human APOL1 expression. For these studies we validated commercial anti-APOL1 antibodies for specificity which may have contributed to prior discrepancies on kidney expression patterns. In addition, APOL1 transgenic mouse models were made proteinuric by intercrossing with a model of HIV-associated nephropathy (HIVAN), a CKD strongly associated with carriage of APOL1 risk alleles, to determine if proteinuria would change the appearance of APOL1 protein in tubular epithelial.

Materials and methods

Human tissue and mouse models

Formalin-fixed, paraffin-embedded human kidney (n = 4) and liver (n = 3) tissue from normal margins of cancer resections were obtained from the Cleveland Clinic Lerner Research Institute Biorepository. Three transgenic mouse lines expressing a 47 kb human genomic fragment in a bacterial artificial chromosome (BAC) encompassing the promoter and coding regions of the human APOL1 gene for each G0, G1, or G2 alleles have been previously described [17, 18]. Each of the BAC-APOL1 transgenic lines were ≥10 generations backcrossed to FVB/Nj, a genetic background susceptible to HIVAN. The mouse HIVAN model used to induce proteinuria was the Tg26 HIVAN4 congenic [19] that develops proteinuria and progressive focal segmental glomerulosclerosis as the parental Tg26 model (Jackson Laboratory #22354) but disease progression is slower. For all studies, kidney disease was monitored weekly after weaning by measuring proteinuria (i.e. amount of protein in spontaneously voided urine) by urinalysis using diagnostic dipsticks (Uristix, Siemens Healthcare). The IACUC-approved humane endpoint for kidney disease in this model was proteinuria reaching 4+ on dipstick. No animal reached this humane endpoint before the
predetermined study endpoint, as this study required an early stage of renal dysfunction (proteinuria on dipstick of 2+ to 3+) as functioning kidneys still capable of filtration and reabsorption were needed. All animals maintained normal weights and exhibited typical grooming and activity levels, and were not in pain or distress during the study. All animals were monitored twice a week by veterinary technicians not associated with this study for overall health and well-being, and no animals required analgesics or other supportive care. Each BAC-APOL1 transgenic mouse line was intercrossed with the HIVAN4 mouse model and were sacrificed at 8–10 weeks of age when their kidney disease progressed to proteinuria levels of ≥2+ by urine dipstick (G0 n = 15, G1 n = 11, G2 n = 9). Terminal urine, blood, and tissue collections were performed under deep isoflurane anesthesia followed immediately (while still under anesthesia) by euthanasia using cervical dislocation. Albuminuria was assessed by polyacrylamide gel electrophoresis of 1 μl urine, followed by Coomassie staining. Use of human tissue was reviewed and approved by the Cleveland Clinic IRB (IRB-06-050). Informed consent was waived because tissue was considered discard and no identifiable data were collected. All animal studies were reviewed and approved by Institutional Animal Care and Use Committees at the Cleveland Clinic and Case Western Reserve University (IACUC-2430).

**Tissue immunofluorescence**

Formalin-fixed, paraffin-embedded kidney tissue sections were subjected to antigen retrieval as previously described [8]. Antibodies used to examine APOL1 expression in this paper was a rabbit anti-human APOL1 (Sigma, HPA018885, lot E105260, 1:400 dilution). Numerous lots of this Sigma rabbit polyclonal in addition to other commercial monoclonal antibodies were also evaluated and the results are summarized in Supplemental Table 1A-1C and Supplemental Figure 1 in S1 Data. Of note, many of these polyclonal antibody lots have been exhausted and are no longer available from Sigma. Other primary antibodies include: rabbit anti-mouse APOA1 (ThermoFisher, 1:500 dilution), goat anti-mouse CD31 (R&D Systems, 1:200 dilution), guinea pig anti-nephrin (USB, 1:200), and mouse anti-GLEPP1 (gift of Roger Wiggins, 1:50). FITC-labeled Lotus tetragonolobus lectin (Vector labs) was used to label proximal tubule cells as previously described [8]. For testing of commercial antibodies against human APOL1, kidney tissues were fixed using a variety of methods and paraffin embedded for immunohistochemistry using various antigen retrieval methods. Details for each of these processing methods are provided in the Supplemental Detailed Methods in S1 Data.

**APOL1 gene and protein expression**

Plasma APOL1 protein concentration was determined using the Meso Scale Discovery electrochemiluminescence immunoassay as described previously [5]. Concentration was determined relative to a known liquid chromatography-mass spectrometry calibrated human high density lipoprotein solution. Serum APOL1 and APOA1 protein levels were compared using Western blotting as previously described [20].

**mRNA in situ hybridization**

APOL1 gene expression was examined in mouse and human kidney and liver tissue using mRNA in situ hybridization. The manual RNAscope in situ hybridization kit (ACDBio) was used for formalin-fixed, paraffin-embedded tissue following kit instructions for either single probe or dual probe detection. Probes included human APOL1 (catalog number 439871), murine nephrin (Nphs1, catalog number 433571), and murine CD31 (Pecam1, catalog number...
Pretreatments were 15 minute boiling and 30 minute protease digestion. The in situ hybridization signal appears as dots; one dot per ten cells is expected background.

Results

Several commercial anti-human APOL1 antibodies (Supplemental Table 1A in S1 Data) were examined for specificity to human APOL1 using tissue or protein extracts from human and mouse kidneys and cells. Since mice do not have an ortholog of human APOL1, murine cells and tissues should not be immunoreactive to antibodies against human APOL1. In Western blotting, most commercial monoclonal and polyclonal antibodies were able to detect APOL1, although several weakly detected APOL1 with stronger detection of non-specific proteins (gels bands that did not coincide with the molecular weight of APOL1, Supplemental Table 1B in S1 Data). Most antibodies detected additional non-specific proteins, which differed depending on the source of protein extracts (Supplemental Table 1B in S1 Data). Some of these non-specific gel bands could be eliminated with pretreatment of protein extracts with deglycosylating enzymes (not shown) suggesting epitope recognition was dependent on glycans. In immunohistochemistry testing, wild-type mouse kidney used as a negative control was compared with APOL1 transgenic mouse kidney (Supplemental Table 1C in S1 Data). Many of the anti-APOL1 antibodies erroneously detected proteins in wild-type mouse kidney, including very strong immunostaining in tubules (Supplemental Figure 1 in S1 Data). Based on these validation studies, we selected a Sigma polyclonal antibody lot with limited off-target detection to examine APOL1 expression patterns.

In human kidney, APOL1 protein was detected by immunofluorescence in glomeruli but not tubules (Fig 1A). Similar expression patterns in human kidney were observed using mRNA in situ hybridization. APOL1 mRNA was present in glomeruli, peritubular capillaries, and larger vessels of the kidney, but not in any tubule segment (Fig 1B). A prior study of human liver transplant recipients established that circulating APOL1 protein is largely produced by the liver [21]. In human liver, APOL1 protein and mRNA expression patterns were similar, with expression detected in hepatocytes and vascular endothelia. In mice, APOL1 expression was qualitatively lower in zone 1 and 2 hepatocytes compared to zone 3 hepatocytes, whereas in human, hepatocytes in all three zones were similar in expression level (Supplemental Figure 2 in S1 Data). The mouse liver tissues were from healthy adults, whereas the human liver tissues were normal margins from cancer resections that also had histopathologic evidence of steatosis, potentially contributing to this difference.

The observed expression pattern in human tissue was confirmed in three transgenic mouse lines expressing a human BAC encompassing the entire APOL1 genomic region for either the G0, G1, or G2 alleles [17, 18]. APOL1 protein was abundant in podocytes and also was present in endothelial cells of glomerular capillaries, peritubular capillaries, and endothelia of larger vessels (Fig 2A). No APOL1 protein was detected in the proximal tubule or any other tubular segment (Fig 2B). Also similar to humans, the BAC-APOL1 transgenic mice had abundant APOL1 in blood (Fig 3), and expressed APOL1 protein and mRNA in liver hepatocytes (Supplemental Figure 2 in S1 Data). In the kidney, this circulating APOL1 protein also could be detected in blood trapped in vascular spaces (Fig 2B). There was no difference in the APOL1 expression patterns between the APOL1-G0, -G1, or -G2 expressing mice (Fig 2B), and is consistent with previous studies in human biopsies from patients with different APOL1 genotypes [8, 10, 22]. The APOL1 protein expression patterns also were confirmed using mRNA in situ hybridization (Fig 4). All three APOL1 genotypes were examined but there were no differences based on APOL1 genotype. Consistent with human kidney (Fig 1), APOL1 was expressed in podocytes (cell type confirmed with co-labeling with Nphs1) and vascular endothelia (cell type...
confirmed with co-labeling with Pecam1), including glomerular capillaries, peritubular capillaries, and larger vessels. APOL1 expression was not detected in proximal tubules or any other tubular segment.

None of the BAC-APOL1 mice spontaneously developed proteinuria, which is consistent with the original description of these mouse models [17, 18], but developed heavy proteinuria when intercrossed with a model of HIV-associated nephropathy (Fig 5). Using immunofluorescence, non-specific anti-APOL1 antibody immunostaining was observed in the wildtype and HIVAN mouse kidneys, mostly in Bowman capsule (Fig 6A). In the intercrossed mice with proteinuria, APOL1 protein was evident in glomeruli, but no APOL1 protein was detected in filtrate or within proximal tubules (Fig 6B). By Western blotting, APOL1 could not be detected in voided urine of proteinuric mice (data not shown). There also was no difference in the pattern of APOL1 expression between the proteinuric APOL1-G0, -G1, or -G2 expressing mice. These same mouse kidneys were immunostained for APOA1, an apolipoprotein that is filtered, as a positive control for vascular distribution and proximal tubule reabsorption.
patterns. Similar to APOL1, APOA1 in plasma was readily detected in glomerular capillary lumens. However, since APOA1 is filtered it also was present in protein reabsorption droplets at the proximal tubule brush border (Fig 6C). In the setting of proteinuria, APOA1-containing
reabsorption droplets increased in number and size at the proximal tubule brush border. A similar pattern in the proximal tubule was not observed with APOL1.

**Discussion**

Antibody specificity has been recognized as one of the most significant challenges impacting reproducible research [23]. We and others had originally reported APOL1 protein is present in proximal tubules of normal and diseased subjects [8, 9]. However, our continuing work with other anti-APOL1 antibodies and using mRNA *in situ* hybridization indicated APOL1 was not expressed in proximal tubules [10]. A remaining possibility is that the APOL1 protein in blood is filtered and reabsorbed, resulting in the appearance of APOL1 protein in proximal tubules. Using *in situ* methods that do not rely on antibodies, along with the newly available human BAC-APOL1 transgenic mice, neither APOL1 protein nor APOL1 mRNA could be detected in tubular epithelia. In the setting of proteinuria, APOL1 also was not filtered. In our hands, the Sigma rabbit polyclonal antibody consistently recognizes human APOL1 but has lot-to-lot differences with recognition of other proteins. The Sigma rabbit polyclonal lot used in studies here does not replicate the strong proximal tubule staining observed in prior lots sold under the same catalog number. Although monoclonal antibodies would eliminate the variation inherent in polyclonal antibody production lots, the commercial monoclonal antibodies tested had significant non-specificity or poor sensitivity for APOL1. A recent report describing the development and validation of a large number of monoclonal antibodies against human APOL1 also did not identify proximal tubule staining in human kidney [24].

Evidence against proximal tubule expression of APOL1, or a role for the proximal tubule in the APOL1-associated CKDs, is accumulating from several other groups. A study using similar humanized APOL1 transgenic mouse models created with fosmids also did not find APOL1 expression in the proximal tubule using both protein and mRNA detection methods [25]. Use of transgenic models with inducible APOL1 expression that restrict expression to either podocytes or tubules found proteinuria and renal pathology occurs only when APOL1 is expressed in podocytes, and not in the tubule [6]. As corroborating evidence in humans, several studies surveying the human urine proteome [26–30] did not identify APOL1 protein in urine, also indicating APOL1 is not filtered in any detectable quantity.

Our studies do not rule-out the possible release of small amounts of APOL1 into the primary filtrate from podocytes either by secretion or passively release when a podocyte dies and
Fig 4. APOL1 mRNA expression in the BAC-APOL1 transgenic mouse models. Duplex mRNA in situ hybridization for (A-D) APOL1 and nephrin (Nphs1) gene expression and (E-K) APOL1 and CD31 (Pecam1) expression to identified podocytes or endothelial cells respectively. APOL1 expression was detected in peritubular capillaries (“1” arrows), arterioles (“2” arrows), glomerular capillaries (“3” arrows), podocytes (“4” arrows), and larger (interlobular) arteries (“5”) and veins (“6”) but not proximal tubules (“PT”) or any other tubular segment. Images shown are from BAC-APOL1-G0 and BAC-APOL1-G1 mice.

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lyses. Unlike the APOL1 expressed and secreted by hepatocytes, the major APOL1 transcript in podocytes does not have a complete signal peptide [15], and there is no conclusive evidence that podocytes secrete APOL1. Alternatively, some studies observed the disease-induced high levels of APOL1 can also produce rare alternatively spliced isoforms with different signal peptide sequences possibly permitting secretion [24, 31–33]. These potential alternative sources of APOL1 in filtrate would have been below limits of detection in the assays we and other investigators have used in kidney tissue and urine, and would be an unlikely explanation for the robust APOL1 expression previously reported in proximal tubules. In addition, it is unclear if this potential low level of podocyte-derived APOL1 in filtrate would be of physiologic significance, considering that in humans, basal levels of APOL1 expression are not associated with CKD. Our studies also cannot rule-out possible differences in APOL1 expression between humans and the BAC-APOL1 transgenic mice. The function of podocytes and proximal tubules in blood filtration and reabsorption are fundamentally similar between humans and mice, however there are acknowledged limitations of using mice with regards to replicating human glomerular kidney diseases [34].

The work presented here and other published studies have shown significant similarities between humans [8–10, 24] and transgenic mouse APOL1 expression patterns [17, 18, 24]. A reproducible and consistent observation from these combined studies is expression of APOL1 in podocytes and in endothelial cells of glomerular capillaries, peritubular capillaries, and larger blood vessels. In addition, observations here indirectly support conclusions from prior studies [4, 5] that circulating APOL1 protein is unlikely to contribute to kidney disease pathogenesis as it is not the source of kidney-localized APOL1. Evaluating the contribution of podocyte- and endothelial-expressed APOL1 is a logical focus for future studies examining the mechanism for APOL1 risk variant contributions to CKD pathogenesis.

Fig 5. BAC-APOL1 transgenic mouse models developed similar proteinuria when intercrossed with the HIVAN mouse model. A. Example of proteinuria in single and dual transgenic mice assessed by gel electrophoresis of urine (Coomassie stain). Arrowhead marks albumin, and additional low molecular weight urinary proteins (asterisk) are a normal finding in mice. B. Western blot of mouse serum in the same single and dual transgenic mice showing maintenance of high levels of serum APOL1 protein in the setting of proteinuria. APOA1 Western blot as a control for a common serum protein that is freely filtered. Representative blot is shown; number of animal examined in each genotype group were: wildtype, n = 3; HIVAN4, n = 6; G0 x HIVAN4, n = 6; G1 x HIVAN4, n = 3; G2 x HIVAN4, n = 4.

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Fig 6. APOL1 does not appear in proximal tubules in proteinuric BAC-APOL1 transgenic mice. A. Control immunostaining in non-transgenic (wildtype) and proteinuric HIVAN4 mice for APOL1 along with fluorescent *Lotus tetragonolobus* ("LT") lectin binding to demarcate the proximal tubule brush border. Since wildtype and HIVAN4 mice do not have APOL1, the staining observed in parietal cells (arrows) is artifact. B. Immunostaining for APOL1 in proteinuric BAC transgenic mice of each APOL1 genotype (representative images are shown, number of animal examined in each genotype
group were the same as for Fig 5). Images show proximal tubules at the transition with Bowman capsule. The boxed region is magnified below each panel along with the isolated fluorescent channels shown in black and white. 

C. Positive control immunostaining for a filtered lipoprotein, APOA1, and fluorescently-labelled *Lotus tetragonolobus* ("LT") lectin. An APOL1-G0 mouse and an APOL1-G0 x HIVAN4 dual transgenic mouse with proteinuria is shown; below each respective color panel is the individual fluorescent channels (in black and white) of the boxed region for either LT lectin or APOA1. White arrows mark glomerular capillaries containing circulating APOA1 protein within capillary lumens, red arrows denote APOA1 in protein reabsorption droplets at the brush border of proximal tubules. Scale bar = 40μm.

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**Supporting information**

*S1 Data. Supplemental figures and tables.*

(PDF)

*S1 Raw images. Original, uncropped gel images.*

(PDF)

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