Abstract. Tubular microcyst formation is a prominent histopathologic feature of HIV-associated nephropathy (HIVAN), but its pathogenesis is unknown. HIV-1 has recently been shown to infect renal tubular epithelial cells in patients with HIVAN. In addition, HIV-1 gene expression in renal epithelial cells has been shown to cause a renal disease that is identical to HIVAN in HIV-1 transgenic mice. In these studies, immunohistochemistry for tubular segment-specific markers and mRNA in situ hybridization for HIV-1 was used to determine which tubular segments develop microcysts and which segments express HIV-1 in the kidneys of transgenic mice and patients with HIVAN. It was found that microcysts involve multiple nephron segments in both patients with HIVAN and HIV-1 transgenic mice. Furthermore, HIV-1 infection in HIVAN and HIV-1 transgene expression also occurs in multiple segments of the nephron. These data support a direct role for HIV-1 infection of renal epithelial cells in the pathogenesis of microcyst formation in patients with HIVAN.

HIV-associated nephropathy (HIVAN) is the third most common cause of end-stage renal disease (ESRD) in African Americans aged 20 to 64 yr. It is also the most common cause of ESRD in HIV-1 seropositive individuals (1). HIVAN occurs almost exclusively in African Americans and is typically diagnosed late in the course of HIV-1 infection (2,3), although earlier cases are increasingly being recognized (4,5). Before the introduction of highly active antiretroviral therapy, renal prognosis was poor, with most patients progressing to ESRD within 4 mo (2). The diagnosis of HIVAN cannot be made by clinical criteria alone and requires a renal biopsy. Diagnostic changes upon histologic examination of the biopsy include focal segmental glomerulosclerosis, usually of the collapsing variant, combined with microcystic dilation of tubules (Figure 1A) (6–8). Other tubular abnormalities observed in HIVAN include tubular atrophy, tubular epithelial simplification, loss of brush border, and proximal tubular protein and lipid resorption droplets (7).

The pathogenesis of microcyst formation in HIVAN remains poorly understood. Microcysts are dilated tubules that have at least three times the external diameter of normal tubules. We have reported previously that renal tubular epithelial cells are infected by HIV-1 in HIVAN (9). We have also demonstrated that HIV-1 gene transcription ceases as tubules dilate and the epithelium becomes flattened and atrophic (10). The nephron segments that become infected by HIV-1 and develop microcysts in HIVAN, however, remain unknown. If HIV-1 infection and microcyst formation were found to occur in the same tubular distribution, this would provide suggestive evidence for a direct etiologic role for HIV-1 infection in the formation of renal tubular microcysts.

In this study, we have used immunohistochemistry for tubular segment-specific markers and mRNA in situ hybridization for HIV-1 to identify the specific segments involved in microcystic tubular disease in HIVAN. We show that in both human HIVAN and the HIV-1 transgenic mouse model, HIV-1 expression and microcyst formation involve multiple nephron segments and that the distribution of HIV-1 expression and microcyst formation are similar.

Materials and Methods

Human and Murine Kidney Tissue

The development of the transgenic mouse line TgN(pNL43 d14)26L has been reported previously (11,12). The proviral transgene, pNL4–3:d1443, encodes all HIV-1 genes except gag and pol. The provirus is replication-defective, and the mice are noninfectious; however, heterozygotes develop proteinuria, renal failure, and renal disease that is histologically identical to HIVAN in humans. Homozygous mice develop a wasting phenotype and seldom survive to weaning (13,14). This murine model of HIVAN has been characterized extensively (11,12,15). The distribution of HIV-1 transgene expression has been previously shown to be highest in skin, tail, and muscle, with lower levels present in kidney and intestine and little expression in liver as detected by Northern blot analysis (16). Transgene expression is detectable by in situ hybridization in several renal epithelial cell types (glomerular visceral and parietal epithelial cells and tubular epithelial cells) but not in mesangial or endothelial cells (15).

All studies using mice were approved by the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine.

Human biopsy material was collected previously from patients with HIVAN after informed consent under a protocol approved by the Mount Sinai Institutional Review Board.

Human (n = 6) and normal (n = 4) and transgenic (n = 4) mouse
kidney tissue was fixed in 4% paraformaldehyde at 4°C for 2 to 4 h. Tissue was embedded in paraffin and stored at −80°C to prevent RNA degradation.

Light Microscopy and Alkaline Phosphatase Staining
Paraffin-embedded sections were dewaxed and rehydrated in a series of graded ethanols. Slides were stained with periodic acid-Schiff or hematoxylin and eosin. Endogenous alkaline phosphatase was detected by incubation in 0.3 mM 4-nitro blue tetrazolium chloride and 0.3 mM 5-bromo-4-chloro-3-indolyl-phosphate in 0.2 mM Tris buffer (pH 9.5) for 5 to 10 min.

Immunohistochemistry
Paraffin-embedded tissue sections (5 μm) were placed onto Superfrost Plus slides (VWR Scientific, West Chester, PA). Slides were dewaxed in xylene and rehydrated in a graded series of ethanols. Slides were then incubated in aqueous 7.5% H₂O₂ to block endogenous peroxidase activity. Slides were then incubated in 2% normal goat or rabbit serum in phosphate-buffered saline (PBS) at room temperature for 40 min. Slides were then incubated for 45 min with one of the following primary antibodies and dilutions: rabbit anti-rat aquaporin-1 (AQP-1), 1:500 (Alpha Diagnostic, San Antonio, TX); rabbit anti-rat AQP-2, 1:50 (Alpha Diagnostic); goat anti-human Tamm-Horsfall glycoprotein, 1:1500 (Cappel Pharmaceuticals, Aurora, OH); or rabbit anti-mouse epidermal growth factor (EGF), 1:200 (Upstate Biotechnology, Lake Placid, NY). Primary antibodies were diluted in PBS containing 2% bovine serum albumin and 2% normal serum from the secondary antibody species. After incubation, slides were washed twice in PBS–0.1% Tween 20 and once in PBS for 5 min each. Slides were then incubated with one of the following biotinylated secondary antibodies: goat anti-rabbit IgG (Vector Laboratories, Burlington, CA) or rabbit anti-goat (Pierce, Rockford, IL). Slides were then washed twice in PBS–0.1% Tween 20 and once in PBS and then incubated for 45 min with avidin-peroxidase (Vectastain elite, Vector Laboratories). Slides were washed once in PBS and twice in Tris-buffered saline for 5 min each. Color development was accomplished by incubating slides with aminoethylcarbazole for 10 to 30 min followed by three 5-min rinses in water. Slides were then counterstained with hematoxylin (Vector Laboratories), rinsed three times in water for 2 min, mounted in Aqua Poly/Mount (Polysciences, Warrington, PA), and photographed under an Olympus BX60 microscope (Olympus, Tokyo, Japan).

In Situ Hybridization
Paraffin sections (5 μm) from mouse and human renal tissue were placed onto poly-L-lysine–coated slides and dried for 2 h at 42°C. In situ hybridization using digoxigenin-labeled riboprobes was performed as described previously (17).

For in situ hybridization in HIV-1 transgenic mice, riboprobes to HIV-1 nef were generated as described previously (15) from plasmids pGM92 and pGM93 (NIH AIDS Research and Reference Reagent Program). These plasmids contain 1.1 kb of the HxB₂ HIV-1 nef coding region in opposite orientations to generate sense and antisense riboprobes. The 1.1-kb riboprobes were randomly cleaved with alkali to an average length of less than 500 bp to enhance tissue penetration.

For in situ hybridization in human tissue, sense and antisense riboprobes to HIV-1 gag were generated from a 359-bp PCR fragment from HxB₂ (nt 1031–1390) subcloned into pGEM-T Easy (Promega, Madison, WI).

Results
Tubulointerstitial Disease in HIVAN and HIV-1 Transgenic Mice
Tubular microcysts are prominent histologic features of HIVAN and the renal disease in HIV-1 transgenic mice (Figure 1). The morphology of the tubular microcysts is similar in HIVAN (Figure 1A) and HIV-1 transgenic mice (Figure 1B).

Localization of Microcysts in HIV-1 Transgenic Mice
To determine which tubular segments develop microcysts, we immunostained kidneys from HIV-1 transgenic mice for

Table 1. Antigens used to identify specific nephron segments

<table>
<thead>
<tr>
<th>Marker</th>
<th>Segment</th>
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<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>Proximal tubule</td>
</tr>
<tr>
<td>Aquaporin-1</td>
<td>Proximal tubule and thin descending limb</td>
</tr>
<tr>
<td>Tamm-Horsfall protein</td>
<td>Thick ascending limb</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>Thick ascending limb and distal tubule</td>
</tr>
<tr>
<td>Aquaporin-2</td>
<td>Principal cells of collecting duct</td>
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</table>
Figure 2. Microcysts involve all tubular segments in HIV-1 transgenic mice. Immunohistochemistry was performed on kidneys from normal mice (left column) and HIV-1 transgenic mice (middle and right columns) using the following tubule segment-specific markers: alkaline phosphatase (Alk phos), aquaporin-1 (AQP-1), Tamm-Horsfall protein, epidermal growth factor (EGF), and aquaporin-2 (AQP-2). Photographic enlargement of tubules from center column are shown in right column. Magnifications: ×200 in A through D; ×400 in E through R.
Figure 3. Patients with HIVAN develop tubular microcysts in the same tubular distribution as HIV-1 transgenic mice. (A) A microcystic proximal tubule expressing AQP-1 is shown. (B) Photographic enlargement of the same tubule reveals apical and basolateral localization of aquaporin-1. (C) A microcyst involving the thick ascending limb (positive for Tamm-Horsfall protein). (D) Photographic enlargement of C. Note that not all cells along the tubule stain for Tamm-Horsfall protein. (E) A microcystic collecting duct expressing AQP-2. (F) Photographic enlargement of the same tubule demonstrates apical localization of AQP-2. Magnifications: ×400 in A, C, E.

Figure 4. HIV-1 transgene expression occurs in tubular epithelia throughout kidneys in HIV-1 transgenic mice. (A) In situ hybridization using HIV-1 nef sense control riboprobe demonstrated no specific staining. However, in situ hybridization with an HIV-1 nef antisense riboprobe (B) revealed staining in tubular epithelia from all regions of the kidney. Magnification, ×100.
tubule segment-specific antigens (Table 1). We chose this method instead of lectin staining because the carbohydrate moieties on the surface of renal epithelial cells to which lectins bind may be altered in the presence of tubular disease (18). Devuyst et al. (19) reported that AQP-1 and AQP-2 retain their tubular segment-specific expression in autosomal dominant polycystic kidney disease (ADPKD) even into end-stage disease. Alkaline phosphatase (20), EGF (21,22), and Tamm-Horsfall protein (23,24) expression have also been characterized in normal kidneys and in cystic renal diseases.

Microcysts were found in proximal tubules (positive for alkaline phosphatase; Figure 2, B and C), thin descending limb (positive for AQP-1; Figure 2, E and F), thick ascending limb (positive for Tamm-Horsfall protein and EGF; Figure 2, H, I, K, and L), and collecting duct (positive for AQP-2; Figure 2, N, O, Q, and R). Cystic proximal tubules were also detected by AQP-1 staining (data not shown). Individual microcysts did not express more than one of these proteins when serial sections were examined (data not shown).

Localization of Microcysts in Patients with HIVAN

Immunostaining for tubule segment-specific markers was performed in renal biopsy specimens from patients with HIVAN (Figure 3). Microcysts were found in proximal tubules (positive for AQP-1; Figure 3, A and B), thick ascending limb (positive for Tamm-Horsfall protein; Figure 3, C and D), and collecting duct (positive for AQP-2; Figure 3, E and F). Thus, in HIV-1 transgenic mice and patients with HIVAN, tubular microcysts develop in multiple nephron segments. Interestingly, Tamm-Horsfall staining, when present, was often heterogeneous within microcystic tubules. Despite the heterogeneous expression of Tamm-Horsfall protein, however, HIV-1 expression in cells lining these tubules was homogeneous (data not shown).

Localization of HIV-1 mRNA in Transgenic Mice and HIVAN Biopsies

In HIV-1 transgenic mice, mRNA in situ hybridization using an antisense riboprobe to HIV-1 nef revealed expression of HIV-1 mRNA throughout the kidney (Figure 4). As microcysts expand and the tubular epithelium becomes atrophic, expression of the HIV-1 transgene decreased (10).

In patients with HIVAN, HIV-1 expression by in situ hybridization was also determined. HIV-1 was detected in several, but not all, of the tubules that were examined (Figure 5). Colocalization of HIV-1 mRNA was performed by immunostaining serial sections for tubule segment-specific markers (Figure 5). HIV-1 mRNA colocalized with markers for prox-
nal tubules (positive for AQP-1; Figure 5, A through C), thick ascending limb (positive for Tamm-Horsfall protein; Figure 5, D through F), and collecting duct (positive for AQP-2; Figure 5, G through I). HIV-1 expression was also detected in podocytes (Figure 5E) and interstitial leukocytes (Figure 5I), as has been previously reported (15). Thus, in both the murine model of HIVAN and in patients with HIVAN, HIV-1 gene expression occurred in multiple nephron segments. Epithelial cells lining severely dilated tubules were negative for HIV-1 expression; however, mildly dilated tubules were often positive for HIV-1 as seen in Figure 5, A through C.

Discussion

Studies involving HIV-1 transgenic mice have been valuable in advancing our understanding of the pathogenesis of HIVAN. In this murine model of HIVAN, renal expression of HIV-1 is necessary for the development of HIVAN (15). Similarly, in patients with HIVAN, HIV-1 infects and is expressed in renal epithelial cells (9). The segments of the renal tubule that both develop microcysts and are infected by HIV-1 have remained unclear.

In the present studies, we have shown that microcyst formation occurs in multiple nephron segments in both HIV-1 transgenic mice and in HIVAN biopsy samples. In cystic diseases, such as ADPKD, cysts are derived from all nephron segments (25,26), and in autosomal recessive polycystic kidney disease (ARPKD), cysts are derived predominantly from collecting duct (27). Thus, the distribution of microcyst formation in HIVAN most closely resembles that in ADPKD. This finding also suggests that HIV-1 can infect epithelial cells from all nephron segments.

In ADPKD and ARPKD, tubular epithelial cell proliferation is a key element in the pathogenesis of cyst formation (22,28–30). In HIVAN, increased proliferation occurs in both podocytes and tubular epithelial cells (31). HIV-1 gene expression has been shown to be associated with increased epithelial cell proliferation in HIV-1 transgenic mice (15). In HIV-1 transgenic mice, all normal and proliferating tubular epithelial cells express the HIV-1 transgene. When the epithelium of microcysts becomes flattened and atrophic late in the disease process, however, transgene expression ceases (10). These findings suggest that renal epithelial HIV-1 gene expression induces epithelial proliferation. This temporal relationship in which HIV-1 gene expression is greatest in relatively normal appearing host cells but is lost as microcysts develop may explain why microcysts do not evolve into macrocysts in HIVAN. In addition, the tubular distribution of HIV-1 expression was demonstrated to be similar to the distribution of microcysts in that multiple nephron segments are involved in each case. These findings are consistent with the hypothesis that HIV-1 expression has a direct role in microcyst formation.

In ADPKD, renal epithelial cells lose some characteristics of mature renal tubular cells such as basolateral polarization of the Na,K-ATPase and the epidermal growth factor receptor (17,22,28,32,33). ADPKD tubular cells, however, retain normal expression of other proteins, such as AQP-1 and AQP-2, even into end-stage disease. Similarly, in HIVAN, although Na,K-ATPase expression may be altered (10), proteins, such as AQP-1 and AQP-2, retain their normal patterns of expression. In HIVAN, Tamm-Horsfall protein expression occurs in its normal location, the thick ascending limb of Henle; however, expression ceases in some cells lining microcystic tubules. This may reflect changes in cellular differentiation induced by HIV-1 infection. Thus, in HIVAN as tubules dilate, some proteins retain their normal expression and distribution patterns and others are altered (32). This is similar to the findings in ADPKD.

In summary, we have demonstrated that both microcyst formation and HIV-1 infection of renal tubular epithelial cells occur in multiple nephron segments. The distribution of microcyst formation and HIV-1 infection are similar, which is consistent with a causal role for HIV-1 infection of tubular epithelial cells in microcyst formation. Loss of HIV-1 expression in epithelial cells lining microcysts may cause proliferation to cease, arresting tubular dilation at the microcyst stage. Future studies defining the viral genes responsible for epithelial proliferation and the host responses that limit HIV-1 gene expression will help clarify the molecular mechanisms for microcyst formation.

Acknowledgments

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References

10. Barisoni L, Bruggeman LA, Mandle P, D’Agati VD, Klotman PE: HIV-1 induces renal epithelial dedifferentiation in a trans-