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HIV-1 induces renal epithelial dedifferentiation in a transgenic model of HIV-associated nephropathy

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HIV-1 induces renal epithelial dedifferentiation in a transgenic model of HIV-associated nephropathy.

Background. Human immunodeficiency virus-associated nephropathy (HIVAN) is the most common cause of renal failure in HIV-1–seropositive patients. Recent studies using an HIV-1 transgenic mouse model have demonstrated that expression of HIV-1 in the kidney is required for the development of HIVAN. What has remained unclear, however, is the renal cell type responsible for pathogenesis and the essential pathological process.

Methods. To address these issues, we used a transgenic murine model of HIVAN. We identified the cell types in kidney in which HIV transgene expression occurs using in situ hybridization. We evaluated evidence of proliferation by immunocytochemical analysis using an antibody to Ki-67 and cell type-specific markers, including WT-1, synaptopodin, Na^+,K^+-ATPase, adducin, and desmin. TUNEL assay was used to evaluate apoptosis.

Results. We found that glomerular and tubular epithelial cells express the HIV-1 transgene early in the disease process when renal architecture is well preserved. Transgene expression is lost, however, in tubular epithelial cells when they lose their differentiated cuboidal phenotype. In glomerular epithelial cells, dedifferentiation occurs with reduced expression of WT-1 and synaptopodin, in association with activation of desmin expression. Tubular microcysts also form with mislocalization of Na^+,K^+-ATPase expression to the lateral and apical cellular membranes.

Conclusions. These studies support the hypothesis that the glomerular and renal epithelial cells are the primary targets of HIV-1 pathogenesis in the kidney. The essential pathologic process is dysregulation of the epithelial cell cycle with increased proliferation, apoptosis, cellular dedifferentiation, and altered cellular polarity.

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Human immunodeficiency virus (HIV)-associated nephropathy (HIVAN) is the most common cause of renal failure in HIV-1–seropositive patients and the third leading cause of renal failure in African Americans between the ages of 20 to 64 [1]. Typically, HIVAN occurs late in HIV-1 infection, with a prognosis similar to that of other AIDS-defining conditions [2]. Upon histopathological examination, HIVAN is characterized by the findings of coexistent glomerular and tubulointerstitial disease. The tubulointerstitial changes include microcystic dilation, interstitial fibrosis, and tubular cell proliferation and apoptosis [3, 4]. In both humans and the murine model, glomerular lesions include focal and segmental glomerulosclerosis, glomerular collapse, and podocyte hyperplasia. In HIVAN, as well as idiopathic collapsing focal segmental glomerulosclerosis in humans, podocytes undergo characteristic changes, including loss of the differentiation markers synaptopodin, WT-1, GLEPP-1, and CALLA. Loss of the expression of these markers is usually associated with cellular proliferation [5].

The role of HIV-1 infection in glomerular and tubular pathogenesis is not completely understood, but increasing evidence supports a direct effect of the virus on renal cells either as a result of exposure to viral proteins or direct renal parenchymal infection. Recent studies using an HIV-1 transgenic mouse model have demonstrated a direct etiologic link between HIV-1 expression in kidney and the development of HIVAN [6]. Conaldi et al have recently demonstrated that HIV-1 infection of human renal cells is possible in vitro. They demonstrated that infection of renal cells by HIV-1 could be detected by reverse transcription-polymerase chain reaction (RT-PCR) of *gag* RNA at a low level [7]. In this article, we provide evidence that the most likely cell target for HIV pathogenesis in the murine model is the renal epithelial cell. The importance of this observation is that the cellular targets for HIVAN pathogenesis have not been defined.

METHODS

Transgenic mice

The transgenic mouse line TgN(pNL43d14)26Lab ("Tg26") has been described previously [8]. The transgene contains a 3 kb deletion spanning the *gag* and *pol* genes, rendering the construct nonreplicating and noninfectious. We have previously shown that homozygous and heterozygous mice develop multiple phenotypes, including a progressive renal disease in heterozygotes that has been described in detail by Kopp et al [9]. In the Mount Sinai colony, the transgene insertion site and transgene expression were similar to that from our previously reported colony at the National Institutes of Health (NIH, Bethesda, MD, USA), as observed by restriction fragment length polymorphisms and Northern blotting, respectively. The phenotype of the two colonies is also similar. Preweanling homozygous and heterozygous transgenic mice (ages 4 to 12 days) have no difference in renal pathology when compared with age-matched normal controls, similar to data previously reported from the NIH colony [9]. Homozygous mice seldom survive to weaning, however [10], and as a result, heterozygous mice were used for these studies. Animals were screened for renal disease by measuring proteinuria by dipstick on a scale of trace to 4+.

In situ hybridization

Tissue fixation, riboprobe preparation, and in situ hybridization for HIV-1 mRNA was performed on mouse kidney tissue as previously described [6] using digoxigenin-labeled riboprobes and color development with the "Genius" nucleic acids detection kit from Boehringer Mannheim (Indianapolis, IN, USA). The probe was a 1.1 kb fragment from the 3′ end of the HIV-1 coding region (*nef* gene) obtained from plasmids pGM92 and pGM93 (NIH AIDS Research and Reference Reagent Program). This region is contained within all spliced and unspliced HIV-1 messages, and thus, expression of all HIV-1 genes can be detected. The probe was randomly cleaved with alkali (less than 500 bp fragments) to facilitate tissue penetration.

Morphologic analysis

Kidneys from normal fetal and adult mice and transgenic adult mice were fixed in 10% formalin and were embedded in paraffin. Three-micrometer sections were stained with periodic acid-Schiff base and Masson trichrome. The amount of segmental and global sclerosis and tubulointerstitial damage was measured semiquantitatively (0 = absent; 1+ = <25% of structures affected; 2+ = 25 to 50%; 3+ = >50%).

Apoptosis assay

Terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay was performed as previously described [11]. The biotin label was detected with an avidin-biotin peroxidase system (Vector Laboratories, Burlingame, CA, USA), and color development was with diaminobenzidine.

Table 1. Summary of immunostaining for markers of proliferation and podocyte differentiation by stage of glomerular development

	Vesicular bodies	S-shaped bodies	Capillary loop-stage	Mature glomeruli
Synaptopodin	−	−	++	+++
WT 1	−/+	+	++	+++
Ki-67	+++	++	+	−
Desmin	−	−	−	−

Immunostaining was graded semiquantitatively: −, −/+, +, ++, +++.

Immunohistochemical analysis

Paraffin sections (3 μm) were immunostained using an avidin-biotin immunoperoxidase technique (Vector Laboratories) as previously described [5]. Primary monoclonal antibodies included synaptopodin (Peter Mundel), desmin (Dako Corporation, Carpinteria, CA, USA), adducin α subunit (Dr. G. Bianchi, University of Milan, Milan, Italy), and Na^+,K^+-ATPase α subunit (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA). Primary polyclonal antibodies included WT-1 (C19; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Ki-67 (Novacastra Laboratories Ltd.). Sections were predigested by microwaving for 25 minutes and were incubated overnight with the following antibodies: synaptopodin (1:10), desmin (1:100), WT-1 (1:100), adducin (1:200), and Na^+,K^+-ATPase (1:10). Horse antimouse or goat antirabbit IgG secondary antibodies (1:100 dilution) and the chromogen diaminobenzidine were obtained from Vector Laboratories. Sections were incubated for 30 minutes with avidin biotin complex (Vector Laboratories) and were developed with diaminobenzidine as chromogen.

Semiquantitative analysis of cysts was performed by determining the percentage tubules that formed cysts. In addition, the intensity and location of staining for Na^+,K^+-ATPase were quantitated in cystic tubules by expressing the percentage of cystic tubules with no staining, with lateral weak staining, with basolateral weak staining, or with basolateral strong staining. Staining in individual tubules was generally uniform; percentage differences reflect intertubule variation.

Table 1 summarizes the markers of podocyte differentiation during glomerular development in normal mice that were used to evaluate transgenic kidneys [5, 12–14]. WT-1 is normally expressed diffusely and weakly in condensing nephrogenic mesenchyme and becomes podocyte restricted in the capillary loop stage [5]. The cytoskeletal protein synaptopodin is considered a marker of maturity in podocytes [15], and its expression coin-

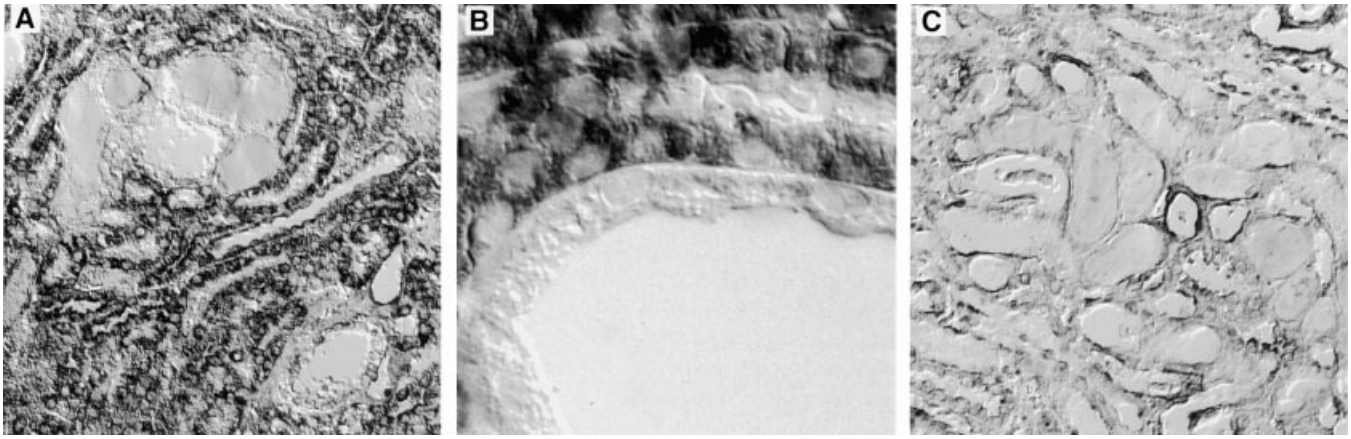

Fig. 1. In situ hybridization for transgene expression in adult, HIV-1 transgenic mouse kidneys. (*A*) Transgenic mouse kidney in the early stages of disease with mild histopathological changes showing loss of transgene expression only in cystic tubules (magnification ×40). (*B*) Transgene expression was lost in cystic tubules with flattened, simplified epithelial cell layer (magnification ×250). (*C*) Transgenic mouse kidney in the late stages of disease, with significant histopathological changes showing overall reduction in transgene expression (magnification ×40).

cides with foot process formation during the capillary loop stage, as shown in Table 1. Its expression is restricted to the basal portion of the podocyte. Simultaneous with the increase in expression of WT-1 and synaptopodin in podocytes, Ki-67 expression, a cell cycle antigen that is detected in 90% of the S-shaped bodies, is lost [16]. Nuclear staining for Ki-67 is absent in glomeruli and tubules of the adult kidney. Desmin, a component of intermediate filaments, is not present in podocytes of normal glomeruli at any stage of development.

Table 2. Comparison of immunostaining between transgenic and normal mice for markers of proliferation and podocyte differentiation

	Normal adult mice	Transgenic adult mice late disease
Synaptopodin	+++	−/+
WT 1	+++	−/+
Ki-67	−	+++
Desmin	−	+++

Immunostaining was graded semiquantitatively: −, −/+, +, ++, +++.

RESULTS

Morphologic microscopic analysis

On gross pathologic examination, kidney size of heterozygous transgenic mice was increased when compared with nontransgenic controls. In heterozygous mice, histologic renal abnormalities were evident by six weeks of age and ranged from minimal involvement in animals, with 1+ proteinuria to extensive glomerular and tubulointerstitial changes in animals with severe edema and massive proteinuria (4+). Affected glomeruli exhibited variable degrees of glomerular sclerosis with Bowman's space often filled by hypertrophic epithelial cells forming pseudocrescents. Microcystic tubulointerstitial disease was readily apparent. The cysts were initially seen at the corticomedullary junction and progressed to involve the entire cortex.

Transgene expression in mice

Expression of viral mRNA was identified by in situ hybridization in a transgenic mouse with early and advanced disease (Fig. 1). Expression of HIV-1 was not uniform and varied greatly depending on the extent of the local disease. In renal tubules, expression was qualitatively higher and more uniform in noncystic or early cystic tubules (Fig. 1A). Transgene expression was greatly reduced or absent in larger cystic tubules lined by flattened epithelial cells (Fig. 1B). In end-stage kidneys, the expression of the transgene was significantly reduced (Fig. 1C).

Analysis of markers of differentiation and proliferation

Since transgene expression was observed predominantly in glomerular and tubular epithelial cells with little or no expression in mesangial cells, we evaluated markers of differentiation and proliferation in these same cell types. The results with the glomerular markers are summarized in Table 2. Comparisons were made between normal animals and transgenic animals with advanced disease. As shown in Figure 2A, synaptopodin was strongly expressed in the glomeruli of normal adult mouse kidney. Synaptopodin expression was significantly reduced in adult transgenic mice with advanced renal disease (Fig. 2B). Furthermore, the distribution and organization of synaptopodin appeared qualitatively different in the transgenic podocytes (Fig. 2 C, D). Similar to synaptopodin expression, WT-1 expression was also

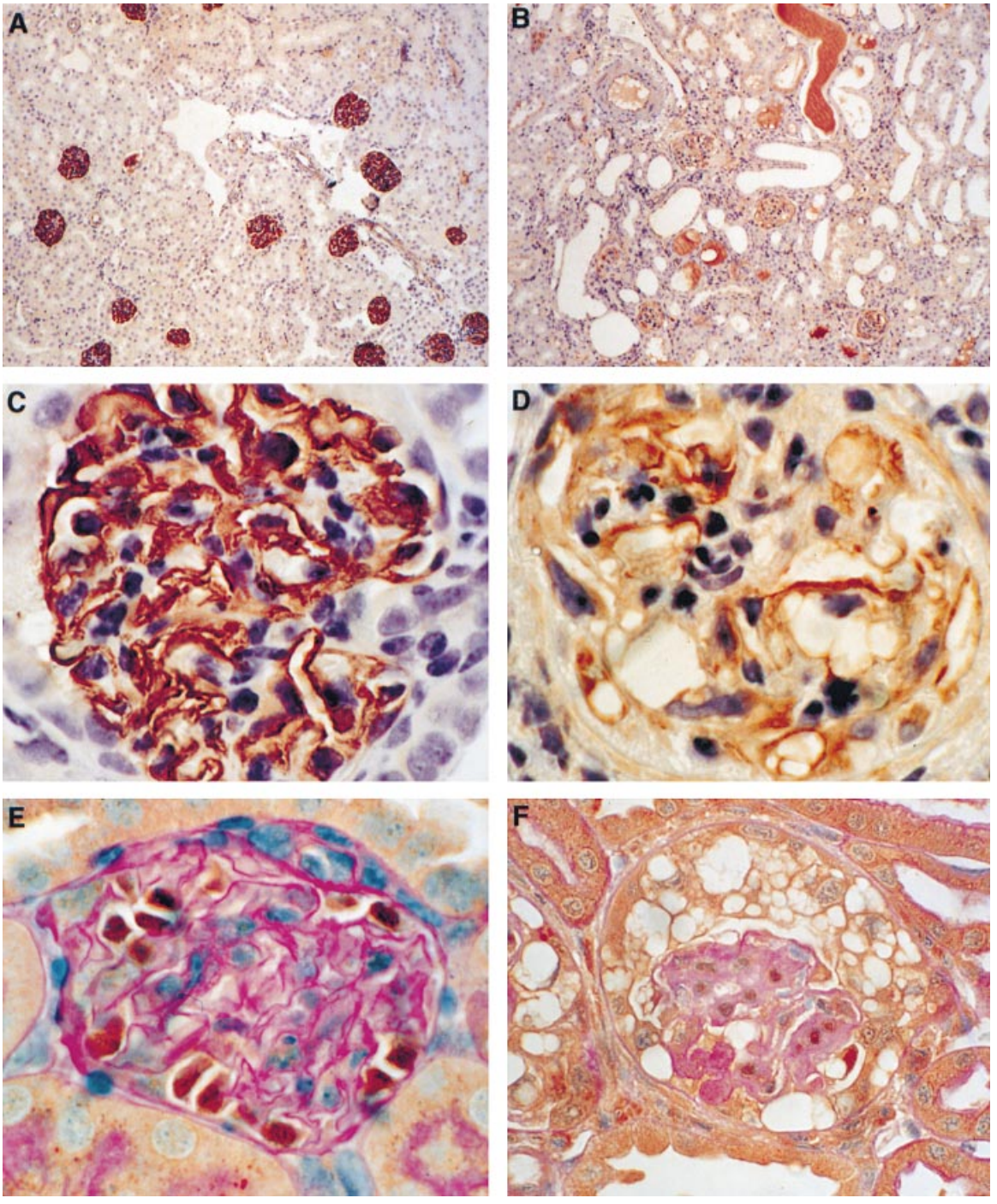

Fig. 2. Immunolocalization for markers of podocyte differentiation, synaptopodin, and WT-1 in normal (*A*, *C*, and *E*) and HIV-1 transgenic mouse kidneys (*B*, *D*, and *F*). Positivity is shown as dark brown staining (counterstaining with hemotoxylin for synaptopodin and periodic acid-Schiff for WT-1). Synaptopodin was strongly expressed in all the glomeruli in normal adult kidney (A; magnification ×25). Higher magnification of the same field showed that synaptopodin was localized in the basal portion of podocyte cytoplasm (C; ×250). In HIV-1 transgenic mice with significant histopathological changes, synaptopodin expression was severely reduced (B and D; ×25 and ×250). WT-1 expression was restricted to podocyte nuclei in normal adult kidney (E; ×250) and was lost in the nuclei of podocytes forming pseudocrescents (F; ×250).

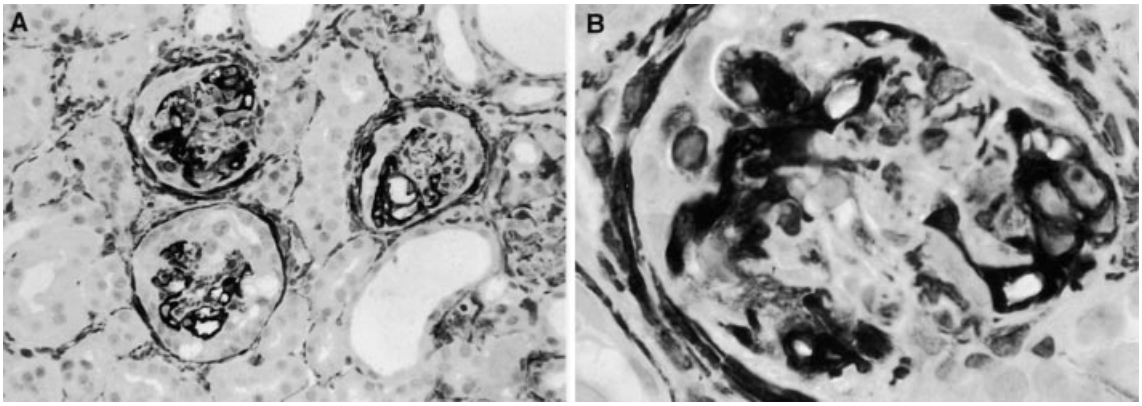

Fig. 3. Immunolocalization for desmin in adult, HIV-1 transgenic mouse kidney. Desmin was strongly expressed in all glomeruli in adult HIV-1 transgenic mice in the late stages of disease (*A*; ×125). Desmin expression was restricted to the inner layer of pseudocrescents formed by podocytes (*B*; ×250).

markedly reduced in the transgenic animals with advanced disease as compared with normal animals (Fig. 2 E, F).

The loss of differentiation markers in the podocytes occurred in the cells that expressed the transgene. Coimmunolocalization for synaptopodin following in situ hybridization showed that synaptopodin expression was disorganized, much reduced, or entirely absent in podocytes that expressed the transgene (data not shown). As also illustrated in Figure 2, the reduction in synaptopodin expression was diffuse in the transgenic kidneys. With colocalization, expression of both the transgene and synaptopodin could be detected in most glomeruli, but those cells that did express synaptopodin had very little expression of HIV-1 mRNA.

The loss of glomerular epithelial differentiation does not represent a return to a fetal phenotype. In animals with end-stage kidneys, desmin was expressed in podocytes and mesangial cells when synaptopodin and WT-1 expression was lost (Fig. 3). Since desmin is not expressed in the podocyte at any time during glomerular development and increased desmin expression occurs in other models of podocyte injury, these findings are more consistent with a dysregulated podocyte phenotype than a return to an earlier developmental stage [13, 17, 18]. In the glomeruli with significant podocyte hyperplasia and pseudocrescents, desmin was strongly expressed in those podocytes attached to the glomerular basement membrane. Desmin was not expressed, however, in the extracapillary cells forming the outer layer of the pseudocrescent (Fig. 3B).

Analysis of tubular epithelial cell differentiation markers in transgenic mice

Approximately 30% of tubules manifested microcystic changes. The polarity of the cystic tubular epithelial cells was evaluated by expression of Na^+,K^+-ATPase. The renal Na^+,K^+-ATPase was located in the basolateral membrane of 100% of the tubules with normal architecture. In microcystic tubules, in contrast, Na^+,K^+-ATPase staining was lost completely in 10%, was located exclusively in the lateral and/or apical membranes in 10%, was weakly associated with the basolateral membrane in 30%, and was normal in 50%. This finding is similar but not identical to adult polycystic kidney disease, in which mispolarization of the tubular epithelium is thought to represent the persistence of a fetal phenotype [19]. In both normal and transgenic mice, Na^+,K^+-ATPase is localized to the basolateral membrane (Fig. 4), suggesting that the tubular cell matures appropriately [20–22]. These data suggest that the epithelial cells do not revert to a fetal phenotype despite the morphologic evidence of cellular dedifferentiation and increased apoptosis and proliferation (described later in this article).

Analyses of epithelial cell proliferation and apoptosis in transgenic and normal mice

Cellular proliferation was evaluated by expression of Ki-67 in glomeruli from normal and transgenic mice with end-stage disease (Fig. 5). Rare proliferating cells were observed in the glomeruli of normal mice that expressed Ki-67 (Fig. 5A). In contrast, transgenic glomeruli had numerous positive nuclei, predominantly in areas in which hyperplastic podocytes formed pseudocrescents (Fig. 5B). Occasional staining for Ki-67 was observed in control tubular epithelium in normals but was increased by 10- to 20-fold in transgenic kidneys, indicating increased proliferation. The amount of proliferation was more evident in early cysts, as compared with larger microcysts in which the epithelium morphologically degenerated to a simplified, flattened cell layer (Fig. 5 C, D).

In addition to cellular proliferation, cell death by apoptosis was examined by TUNEL assay. Apoptotic nuclei were not observed in glomeruli from normal mice (Fig.

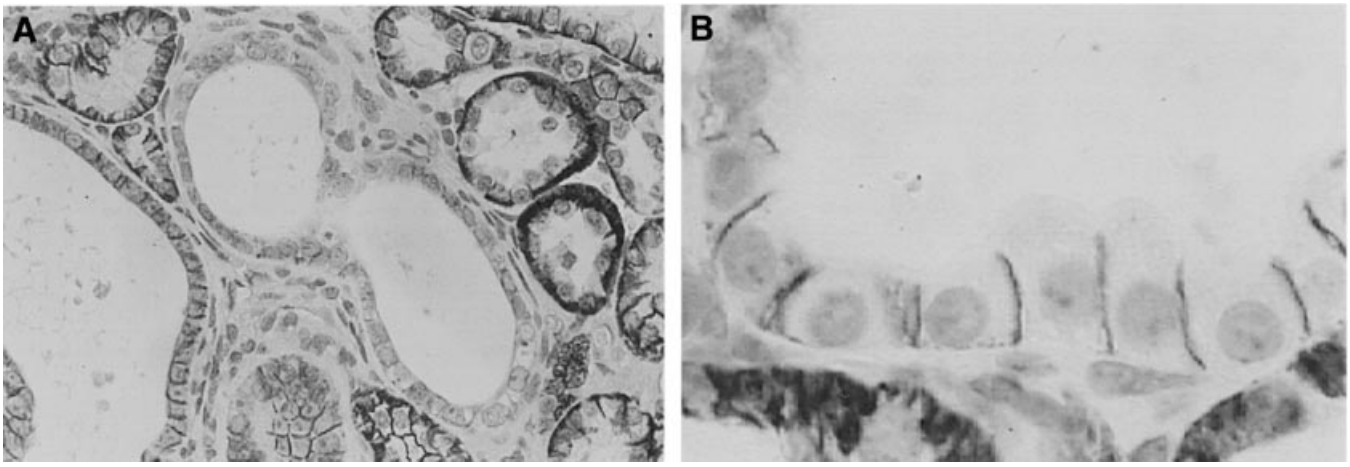

Fig. 4. Immunolocalization for Na^+,K^+-ATPase in microcysts in adult, HIV-1 transgenic mouse kidney. (*A*) Positivity is shown as dark staining material on the basal and lateral membranes of normal tubular epithelium. One cyst was negative, and the other showed predominantly lateral staining with some apical, but none basal (×125). (*B*) Higher magnification (×325) of a cystic tubule shows the absence of basal staining and predominance of lateral localization of Na^+,K^+-ATPase.

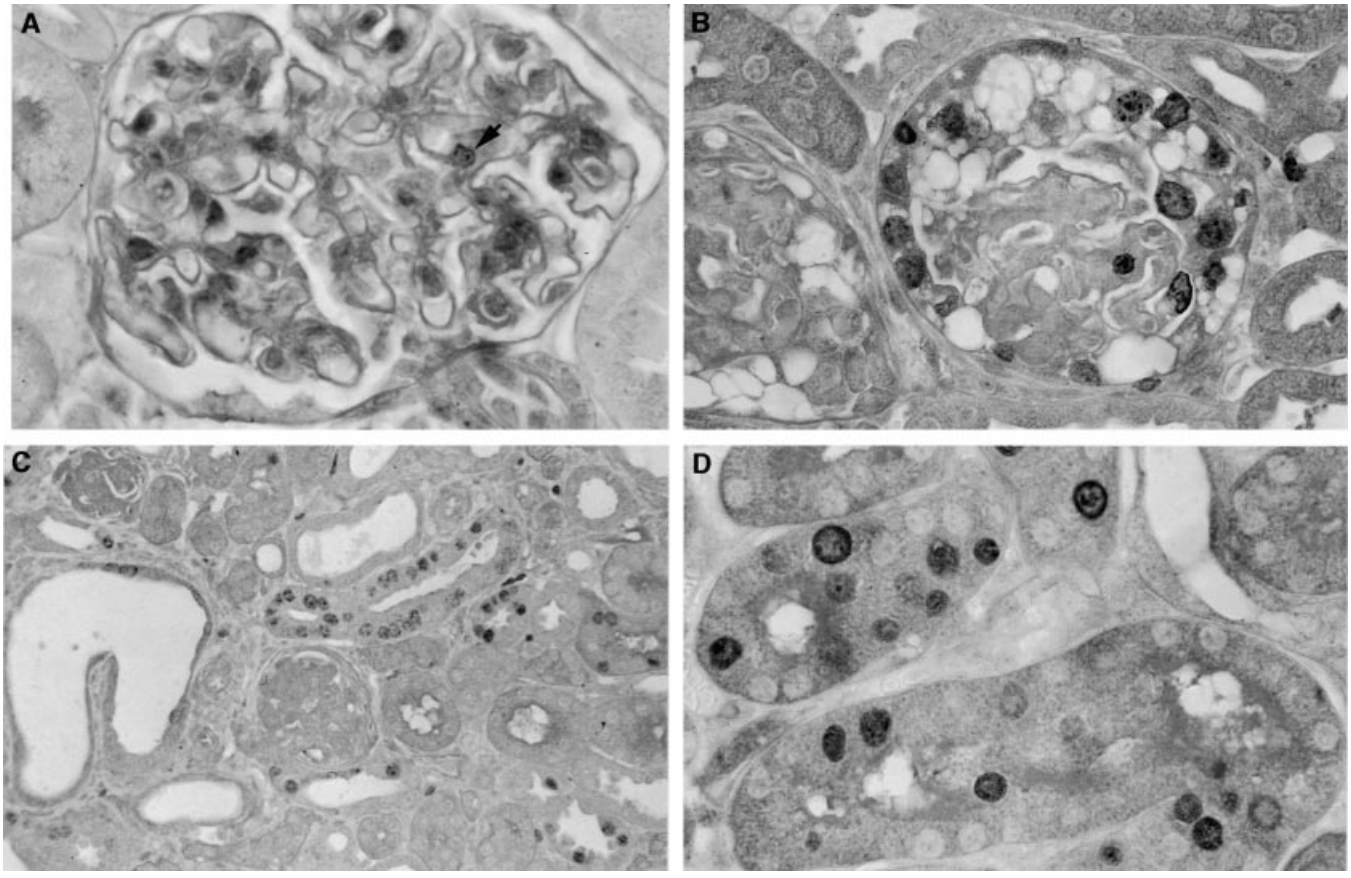

Fig. 5. Nuclear immunostaining for Ki-67 in normal adult (*A*) and transgenic mouse kidney (*B–D*). Rare proliferating cells (indicated by the arrow) were detected in normal glomeruli (A; ×250), whereas podocytes of HIV-1 transgenic mouse showed numerous cells positive for Ki-67 (B; ×150). B is a serial section with the glomeruli shown in Figure 2F. The proliferative index was high also in the tubular compartment of transgenic mouse kidneys and in particular in the precystic tubules (C and D; ×100 and ×250).

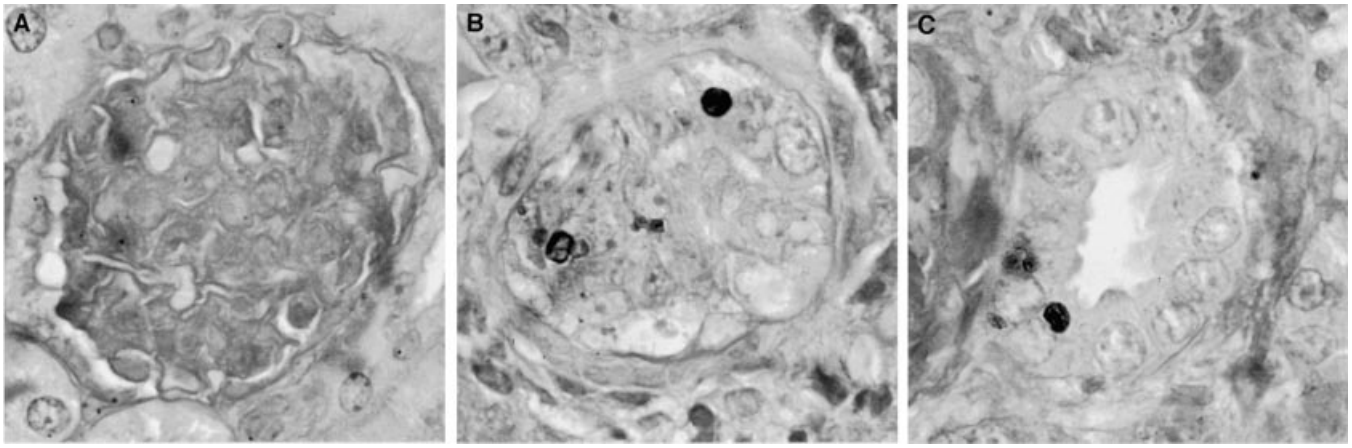

Fig. 6. Analysis of apoptosis by TUNEL assay in normal adult kidney (*A*) and HIV-1 transgenic mouse kidney the in later stages of disease (*B* and *C*). No apoptosis was detected in normal glomeruli (A), whereas rare apoptotic podocytes and parietal epithelial cells were present in sclerotic glomeruli (B) as well as in early tubular cysts (C; ×250).

6A). In transgenic mice, however, apoptotic cells were obvious in podocytes and parietal cells, particularly in sclerotic glomeruli (Fig. 6B). Apoptotic cells were also seen in noncystic tubules (Fig. 6C), as well as in dilated tubules in animals with end-stage renal disease.

DISCUSSION

Many important issues remain to be resolved in the understanding of HIVAN pathogenesis. Previous studies using the HIV-1 transgenic mouse model have shown that the typical features of HIVAN occur in transgenic kidneys transplanted into nontransgenic littermates [6]. This finding suggests that the phenotypic expression of HIVAN in the mouse is due to the intrarenal expression of HIV-1 mRNA and is independent of circulating viral proteins and dysregulated systemic cytokines or growth factors. In the current studies, expression of HIV-1 mRNA in murine renal epithelial cells in vivo was associated with markers of the disease phenotype that have also been observed in humans. Taken together, these two observations in the mouse model suggest that in humans, renal epithelial cells are the likely targets for pathogenesis, although the mechanisms remain to be determined.

The pathogenesis of HIVAN in humans is strikingly similar to the murine model. In both mouse and humans, collapsing focal segmental glomerulosclerosis develops in concert with significant tubulointerstitial disease [23]. The initial histopathological changes observed in humans and the mouse include tubular microcyst formation with severe tubular degeneration and regeneration, which is frequently the most significant component of the disease process, often exceeding the severity of the glomerulosclerosis. Visceral epithelial cells exhibit striking hypertrophy and hyperplasia, filling the urinary space. In the current study, expression of the transgene occurred in areas in which there was coexistent tubular and visceral epithelial cell proliferation. In contrast, in areas of end-stage disease, the expression of the transgene was generally lost or greatly reduced. Thus, HIV expression in renal cells that develop disease is linked to the pathological process of proliferation and loss of differentiation. Late disease does not appear to support viral transcription, and this may explain, in part, the difficulties of detecting viral mRNAs in late-stage biopsies in humans.

In the mouse model, proliferative changes are associated with the loss of the differentiation markers synaptopodin and WT-1. This is similar to observations made in humans with HIVAN, where loss of synaptopodin expression by podocytes is also observed with increased cellular proliferation [5]. Since desmin is expressed in diseased glomeruli from transgenic mice and is not normally observed during development, the proliferative state in murine HIVAN does not appear to be a return to a fetal phenotype; rather, it appears to be a pathological response to HIV-1. Changes in differentiation are also observed in microcystic tubular epithelial cells. In approximately 50% of cysts, normal polarization is lost, manifested by decreased Na^+,K^+-ATPase staining and/or mislocalization of Na^+,K^+-ATPase to the lateral and apical membranes. The mechanism by which HIV-1 gene expression in these cells leads to abnormal polarization is unknown, but it may explain, in part, the formation of microcysts [21].

Renal epithelium appears to be the cellular target for pathogenesis in both humans and mice. Furthermore, expression of the transgene in renal epithelial cells is associated with the development of the disease in the mouse model. Thus, a viable hypothesis emerges that the intracellular expression of HIV-1 in humans also produces the human disease. Intracellular expression

would require that HIV-1 infects renal epithelium, but direct epithelial infection in vivo has not yet been demonstrated. There is precedence for epithelial cell infection in other systems, however. Epithelial cells derived from cervical mucosa have been shown to be productively infected by both patient isolates and laboratory strains of HIV-1 in vitro [24–28]. Although these cells do not typically express CD4, the route of entry is believed to be through a cell-mediated mechanism involving HIV-1–infected monocytes or macrophages [26]. These cervix-derived epithelial cells have been shown to harbor virus for up to eight months [27]. Thus, cervical epithelial cells can be infected, support HIV-1 replication, and potentially function as a reservoir for the virus.

Renal cells may also support HIV-1 infection, although previously published studies have been conflicting [29–33]. Cohen et al were the first to suggest that HIV-1 infection of kidney tissue was possible by demonstrating HIV-1 DNA and p24 antigen in a single tubule from a human renal biopsy specimen by in situ hybridization and immunocytochemistry, respectively [33]. These data were not confirmed, however, and di Belgiojoso et al failed to find evidence of HIV-1 DNA or antigens in a large group of Italian patients with HIVAN [31]. In vitro studies have also been contradictory. Green, Resnick, and Bourgoignie reported that HIV-1_{IIIB}, a cloned laboratory strain, and HIV-1_{MB}, a primary isolate, could infect mesangial and endothelial cells in vitro [29]. However, these investigators were unable to infect glomerular epithelial cells in culture. Alpers, McClure, and Bursten found that mesangial cells were resistant to infection by several cloned HIV-1 strains [30]. More recently, Conaldi et al demonstrated that infection of renal tubular epithelial cells is possible in vitro, but whether this occurs in vivo remains to be determined [7].

Evidence to support renal epithelial infection in vivo has not been definitive. Recent studies have confirmed the presence of HIV-1 in renal tissue by polymerase chain reaction (PCR), but neither the intracellular location nor the infected cell types were elucidated. Kimmel et al reported that HIV-1 could be detected in microdissected tubules and glomeruli, but not renal interstitium using DNA PCR of the HIV-1 *gag* gene [32]. These data suggest that nucleic acid sequences are present in kidney, but again, did not demonstrate their location. An additional confounding feature of that study was that infiltrates from the biopsy material were negative by PCR. The study was performed in an era prior to highly active antiretroviral therapy (HAART) when some infiltrating leukocytes would have been expected to harbor HIV-1, particularly since macrophages and T cells were detected in the interstitium. Recently, Ray et al have shown that viral isolates from children with HIVAN appear to be able to infect tubular epithelial cells isolated from the same patients when cultured in vitro [34]. Like the other in vitro infection studies discussed, however, the level of HIV-1 infection achieved was extremely low, with p24 values very near the lower limits of detection. Taken together, these studies suggest that HIV-1 is present in the kidney but they do not define the cells or tissue location.

In summary, it has been extremely difficult to define the likely renal cellular target for HIV-1 infection; pathogenic mechanisms involving renal mesangial, interstitial, epithelial, and endothelial cells have each been postulated at various times. The current studies clearly support the hypothesis that renal glomerular and tubular epithelial cells are the primary targets for pathogenesis. Furthermore, HIV-1 expression occurs in cells that appear normal, and with disease, expression is lost. These studies do not yet prove a direct causal link between HIV-1 expression and the development of the disease phenotype. They do establish, to our knowledge for the first time, that HIV-1 expression in renal epithelium antedates the development of disease. These studies also suggest by analogy that intracellular expression of HIV-1 in renal epithelial cells (that is, infection) in humans is a plausible although unproved hypothesis for renal pathogenesis. Clearly, the next steps to our understanding of this disease include (*1*) proof that HIV-1 does or does not infect renal cells in humans, (*2*) the definition of the genetic cofactors responsible for the disease in African Americans, and (*3*) the elucidation of the host pathogenic responses to disease that ultimately produce HIVAN in susceptible individuals.

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